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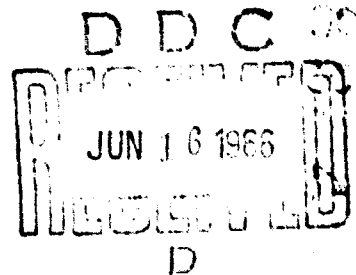
PROPOSAL AND ANNUAL PROGRESS REPORT

A STUDY OF "IMMEDIATE" SENSITIZATION BY ADSORPTION OF
ANTIGENS AND ANTIBODIES IN VITRO

Prepared by

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PHYSICOCHEMICAL AND SEROLOGICAL PROPERTIES OF NONSPASMOGENIC
FRACTION IN TETANUS TOXIN. G. A. Feigen, J. T. Tomita*, and
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Previous studies from this laboratory revealed the existence of a principle in tetanus toxin, physiologically distinct from the centrally acting tetanospasmin, which affected primarily the peripheral neuromuscular junction. Much of the centrally active material could be removed by adsorption with a cerebroside-ganglioside complex leaving a product having a high ratio of peripheral to central potency. Fractionation of the crude material with ammonium sulfate showed the peripheral activity to be concentrated in Fr. IV, which precipitated in the range 70-100% saturated $(\text{NH}_4)_2\text{SO}_4$. The ratio of the peripheral to the central activities was 7.21×10^3 , representing a 60-fold increase over the starting material. Remon titration showed this to be 17 times more potent than the crude material and immunoelectrophoresis showed 12 lines as compared to 20 lines in the crude toxin. The results of physicochemical studies performed to date are: partial molar volume: 0.741, Svedberg Coefficient: $5.5 S_{20}$, η 1%, molecular weight, as estimated by sedimentation equilibrium: $35-40 \times 10^3$ and 16,000 by osmotic pressure. Seventeen amino acid residues were found, the principal ones being aspartic 15%, glutamic 12%, lysine 14%. (Supported by grant-in-aid HE 03693 from USPHS and Contract NONR 225(46) between Stanford Univ. and ONR.

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TABLE OF CONTENTS

	<i>Page</i>
PREAMBLE	3
FUTURE PLANS	5
PROGRESS REPORT	8
SECTION I	8
Anaphylaxis in The Isolated Heart	9
Action of Protein Toxins	10
Tables	13
SECTION II	22
Introduction (Tetanus Toxin)	25
Materials and Methods	26
Materials	26
Purification Methods	27
Material Yield of Purification Procedures	29
Characterization	30
RESULTS	32
Chemical Properties	33
Biological Potency	34
Immunochemical Analysis	36
Ultracentrifugal Analysis	38
CONCLUSIONS	41
TABLES	43
FIGURES	44

PREAMBLE

In the proposal submitted in 1964 we set out as our general aim the study of four systems which were selected to further our understanding of the physiological chemistry, pharmacology, and immunochemistry of sensitization. These systems were:

1. The mechanism of passive sensitization with the ovalbumin-antiovalbumin system.
2. The mechanisms of action of streptolysin,
3. of sea urchin toxin, and
4. of the non-spasmogenic fraction of tetanus toxin.

In retrospect, answers to many of the specific aims suggested as reasonable modes of attack on the several problems have borne fruit and the results either have been published or are being prepared for publication.

Anaphylaxis: Studies in the area of in vitro sensitization are continuing with especial emphasis on the release of various substances from the heart and the gut, and at the present time we are actively studying the problems of the inactivation of the histamine releasing process. Studies on the kinetics of sensitization have been continued with the finding that the rate of sensitization attains a maximum velocity as the antibody concentration is increased, that this maximum velocity varies in a regular way with temperature, which now provides a means of calculating the activation energy of sensitization as distinct from that of histamine release. The lag period of sensitization has been shown to be dependent on antibody concentration and temperature; thus it is from these considerations the lag period turns out to be due to the outcome of the kinetics of sensitization rather than to a separate kind of biological process, as suggested by earlier authors. Studies of in vitro passive anaphylaxis have reached a quantitative refinement such that it is now possible to study hapten inhibition with a ragweed-anti ragweed system and quantitatively to titrate dialyzable haptens prepared by various methods.

Sea Urchin Toxin: In previous studies we described conditions leading to the release of materials such as histamine and various catecholamines from the gut, lung, and heart of the guinea pig; and, we have published our work on the isolation and physical properties of the most active fraction of the toxin. In the present report we show that the active fraction is a proteolytic enzyme which attacks α_2 -globulins to produce a dialyzable kinin, probably a polypeptide, which has properties similar to that of bradykinin.

Streptolysin: Our earlier work had shown that the cardiac irregularities consequent upon the perfusion of streptolysin through the coronary circulation was associated with the release of large quantities of potassium and catecholamines, including serotonin. Direct tests with serotonin were only moderately effective in reproducing the phenomenon. Recent experiments on atrial potentials have shown that the repolarization time of single cells is specifically affected, suggesting an electrical counterpart to the leakage of potassium. This finding is typical of the action of acetylcholine and for this reason the phenomenon was investigated pharmacologically to determine whether, in fact, Ach could be involved. We show that an increase in repolarization rate can be prevented by atropine, and furthermore, that the bath fluid remaining after treatment of atria with the toxin contains material having the pharmacological properties of acetylcholine.

Tetanus Toxin: Finally, perhaps the most successful area of our work has been in the purification, characterization, and mode of action of the non-spasmogenic principle of tetanus toxin. We have succeeded in reducing the number of antigenic determinants from twenty to two by various modes of treatment; we have purified the classical tetanospasmin to the point at which only 1 antigenic determinant is seen in immunoelectrophoresis permitting us to identify the remaining determinant as that of the non-spasmogenic factor. The two products are antigenically, biologically, and physically distinct from each other. The molecular weight of NSP is about one-third that of tetanospasmin, and the ratio of the electrophysiological to the lethal activity is about 10^{10} .

FUTURE PLANS

In vitro sensitization Studies on the process of sensitization will be continued with the view of determining whether the high activation energy of sensitization is entirely the result of site- γ globulin interaction or whether a significant portion of the overall temperature coefficient results from the displacement of competitive, non-immune γ -globulins from the sites in question. For this purpose the γ -globulin will have to be purified even further by chromatographic methods, and after such resolution, to be treated with antigen and the specific precipitate dissociated in the presence of a high salt concentration.

Streptolysin: Further studies on this toxin will be made with a highly purified preparation, Streptolysin A, to determine which of the many effects produced by the cruder preparation, Streptolysin "O", are attributable to the pure substance. These will include additional studies on the atrial action potential, the release of active materials from the heart and gut, and a reassessment of certain of the pharmacological work with respect to blocking agents.

Sea Urchin Toxin: Once additional supplies of toxin are on hand, a pool of material will be prepared by precipitating the toxin in the presence of 2/3 saturated ammonium sulfate. This fraction, in turn, will be further purified by chromatography on DEAE sephadex and the various fractions characterized both chemically and pharmacologically. Up to now the substrate used has been obtained as an ethanol fraction of human or bovine serum having a high content of α_2 -globulins. When the toxin fractions are available in more purified form the "substrate" will also be purified and the activity of each "substrate" fraction determined with respect to every "enzyme" preparation.

Tetanus: Although the other lines of investigation will be continued, the greatest emphasis will be placed on the *isolation, characterization, and mode of action* of the non-spasmogenic principle of tetanus toxin.

A. Chemical and Immunochemical Experiments

1. Isolation and Purification of Fraction IV and its CGC daughter by
 - a. Column chromatography on various adsorbents, at a range of ionic strengths and pH values
 - b. Electrophoresis: on starch block polyacrylamide gel and starch gel.

- c. Crystallization will be attempted from cold MeOH.
- 2. Physical and Chemical Characterization: The active fraction will be characterized with respect to its
 - a. Optical properties
 - b. Equilibrium sedimentation in the ultra-centrifuge
 - c. Osmotic pressure
 - d. Intrinsic viscosity
 - e. Solubility (phase-rule)
 - f. Amino acid analysis
- 3. Immunochemical characterization:
 - a. Immuno-electrophoresis
 - b. Seroflocculation
 - c. Immunodiffusion
 - d. Complement fixation
 - e. Hemagglutination
- 4. Immunological Experiments:
 - a. Production of toxoid for cross-immunization
 - b. Tests for specificity by anaphylactic methods.
 - i. Passive cutaneous anaphylaxis
 - ii. In vitro anaphylaxis

B. Mode of Action

The mode of action has been grossly established as being due to the depolarization of the prejunctional nerve terminals with the associated reduction in the quantum content of the end plate potential. However, it is becoming increasingly apparent that the action of tetanus toxin is not limited to the junctions but may be more wide-spread in its distribution, e.g., on cardiac reflexes and on the pupillomotor apparatus - as reported by Ambache. Although the sites of action of both tetanospasmin and NSP have now been established, their mode of action is not known. For this purpose we propose to carry out certain experiments on the fixation, biochemical action and electrolyte transport.

1. Physiological Chemistry

- a. Ach-ChE system: Since the point of attack of this preparation is essentially on acetylcholine "depots" work will be undertaken to determine the action of the purified compound on well defined acetylcholine-cholinesterase systems in the brain and liver.
- b. Electrolytes: A study will be made of the electrolyte requirements, particularly of the effects of alkaline earth cations on the mode of action of the nonspasmogenic factor.
- c. Temperature: The effects of temperature will be determined to estimate the temperature optimum reversibility and denaturation point.
- d. Inhibitors: A study of a variety of possible inhibitors such as iodoacetate, DFP; activators such as cysteine, and various potentiators such as succinate, and maleate will be considered for this study as well as Krebs cycle intermediates.

2. Active Transport

Since the activity of the toxin appeared to be centered on various electrogenic membranes, pilot experiments were made on the ion transport in the toad bladder according to the technique of Ussing. These experiments were not entirely conclusive but they left an impression that the transport of Na was indeed affected by the toxin. At the time these experiments were made we were severely limited in the amount of purified material available. Since we expect to have on hand adequate supplies of toxin, these experiments will be extended to provide a firm answer to the question concerning the influence of this fraction on electrolyte transport.

3. Tissue Fixation

The action of the toxin appears to have a rather limited ceiling and it is effective in extremely small concentrations, suggesting that the action may be highly specific and limited to certain sites on the cell. An effort to find the locale of these sites will be made with the use of labeled antibodies and the tissue preparations will be examined by radioautography, fluorescence microscopy, and electron microscopy.

The systems will be labeled with

- a. Ferritin
- b. I^{125}
- c. Fluorescein

SECTION I

ANAPHYLAXIS IN THE ISOLATED HEART AND THE ACTION
OF STREPTOLYSIN AND SEA URCHIN TOXIN

I. ANAPHYLAXIS IN THE ISOLATED HEART

A. *EFFECTS OF TEMPERATURE*B. *INHIBITION BY NATURAL HAPTENS*

II. ACTION OF PROTEIN TOXINS

A. *STREPTOLYSIN*B. *SEA URCHIN TOXIN*

1. Enzymological Experiments

2. Chemical Experiments

a. Production of Active Material from Human Globulin

b. Petroleum Ether Extraction

c. Test for Histamine

d. Test for Serotonin

3. Pharmacological Experiments

III. TABLES

ANAPHYLAXIS IN THE ISOLATED HEART

EFFECTS OF TEMPERATURE

In our previous study a comparison was made of the effectiveness of *in vitro* sensitization achieved by perfusion of the whole heart with antibody as against incubating isolated portions of the organ with various concentrations of antibody. Before that could be done it was necessary to determine whether there were any differences in the various cardiac tissues with respect to histamine release and histaminase activity that might affect the results. Briefly, although the aorta turned out to have the highest histamine content per cent dry weight, the atria released almost all of their histamine store on challenge with antigen, and thus accounted for over 50% of the total releasable histamine in the guinea pig heart. The histaminase activity of cardiac tissue--and of the atria in particular--appeared to be very low, permitting us to use this tissue as a model for further studies of the anaphylactic process.

The velocity of histamine release from *in vitro*-sensitized atria is very high and is temperature-dependent. The activation energy in the range 27-37°C is about 20 kilocalories. A further investigation to obtain the temperature optimum showed that minimal histamine release occurs at 27-33.5°C after which it rapidly increases to the maximum, which lies in the region of 41-45°C. At the present time experiments are being conducted to determine the effect of temperature on reversibility of the anaphylactic reaction.

INHIBITION BY NATURAL HAPTENS

The system used in this study consisted of ragweed extract and its rabbit antibody. The hapten was prepared by acid or alkaline hydrolysis of the whole antigen and the hydrolyzed product was subjected to dialysis. The dialyzable material was freeze-dried and used for hapten inhibition tests. Preliminary sensitization tests in guinea pig tissue showed that maximal sensitization was achieved in two hours with rabbit anti-ragweed γ -globulin, containing 1.0 mg of specifically precipitable antibody/mg γ -globulin. The amount of antigen required to produce a 50% response was determined by a von Krogh interpolation of the dose-response curve to antigen; and, in a separate series of experiments, the inhibitory action of the soluble hapten was studied by determining the concentration of hapten required to block the response to the selected dose of antigen. A typical protocol is presented in Table II.

The amount of hapten required for inhibition was surprisingly high, as can be seen from Table III. It was also reversible. In general, the biological assay reflected fairly clearly the evidence obtained from the routine serochemical tests.

The sedimentation behavior of the various allergens and haptens was studied on the analytical ultracentrifuge. The allergens had an

identifiable peak in the range 1-2 Svedberg units. Some preparations had minor peaks. The dialyzable haptens, in most cases, had no recognizable peaks.

ACTION OF PROTEIN TOXINS

STREPTOLYSIN

In the previous report we described the reaction of the whole heart to various doses of streptolysin. This consisted, grossly, of block and eventual cardiac arrest. Associated with these phenomena was the appearance of large quantities of catecholamines, principally nor-epinephrine and serotonin, as well as a washout of potassium. The unexplained phenomenon was that neither serotonin creatinine sulfate nor *n*-acetyl serotonin produced a significant cardiac disturbance, even at high doses. Accordingly, the preparation was simplified by the use of isolated atria rather than the whole heart.

The addition of streptolysin to atrial preparations while intracellular potentials were being recorded with microelectrodes showed that the repolarization time of the atrial action potential was accelerated. (Table IV). Since there was no corresponding effect on the depolarization phase we inferred that this reaction was an expression of the significant potassium leakage which had been determined analytically in previous experiments, and that the latter might have been produced as a consequence of the release of acetylcholine. The possibility that this transmitter was being released was tested pharmacologically. The accelerated repolarization caused by streptolysin could be prevented by the presence of atropine in the bath; conversely, the depolarization was further accelerated by physostigmine. In separate experiments, the bath fluids were dialyzed and the external material was tested on the normal guinea pig gut. The bath fluid produced contractions which could be blocked with atropine and potentiated with physostigmine, supporting the presumptive evidence that acetylcholine was released.

To determine whether the toxin had a general cholinergic effect, streptolysin was tested on the isolated hemithorax of the mouse during impalement with microelectrodes. The result of this experiment was a dramatic increase in the frequency of the random discharge of miniature end plate potentials followed by a depression. These results suggest that the cholinergic response is not limited to the heart alone but might be more general in nature, affecting many different kinds of acetylcholine "depots" in the body.

SEA URCHIN TOXIN

The padicellular toxin of the sea urchin *Tripneustes gratilla* produces cardiac arrest, intestinal contraction, and hemolysis. Preliminary experiments have shown that histamine is liberated when the toxin is applied directly to the tissue or when it is perfused

through the coronaries of the surviving guinea pig heart: the multiplicity of actions of the crude toxin suggested that it might contain several enzymes which were responsible for the diverse reactions observed. The most active fraction of the crude toxin, as estimated by LD₅₀ (intravenously) in mice, was precipitated in the presence of 65 per cent saturated ammonium sulfate. Ultracentrifugal analysis showed only a single peak having a sedimentation constant of 2S.

Various lines of evidence suggested to us that this fraction might have the properties of a proteolytic enzyme and that, in fact, its lethal effect could be attributed to an attack on certain plasma proteins with the consequent formation of active plasma kinins. Accordingly, various purified proteins were tested as substrates for this material. These were ovalbumin, bovine serum albumin, and serum globulin. The experimental prototype was the exposure of a given concentration of protein to the action of the toxin for a constant time. The reaction was terminated at a standard time by heating. The reaction mixture was dialyzed and the external fluid tested for activity on the guinea pig gut.

Enzymological Experiments

Of the various substances tested only the serum globulins were found to be useful as substrates. The experiment was repeated to determine which fraction of toxin had the greatest potency as an enzyme. The experimental results showed that the fraction precipitating in the presence of 2/3 saturated ammonium sulfate had the greatest activity in this respect. In order to characterize the reaction according to the accepted enzymological methods it was necessary to show that the system obeyed the usual Michaelis-Menten kinetics. For this purpose the velocity of production of active material was studied at a constant "enzyme" concentration for a range of concentrations of serum globulin. The velocity constants were calculated from the time-courses of the several reactions. The results of a typical experiment are given in Table VII.

The nature of the active material is not known but is presumed, from preliminary work, to be a polypeptide. The pharmacological behavior of the active material, summarized below, suggests that it has properties similar to that of bradykinin.

Chemical Experiments

Production of Active Material from Human Globulin: Active material was produced for pharmacological and chemical tests by heating 9 ml of a protein fraction, rich in human α_2 -globulins, with 9 ml of a solution of sea urchin toxin, isolated by precipitation in the presence of 65 per cent saturated ammonium sulfate. The concentration of the globulin was 0.81 mg/ml and that of the enzyme 1.10 mg/ml.

The reaction was allowed to develop for 20 minutes after which 17.5 ml of the reaction fluid was transferred to sausage casing and dialyzed against cold 1% NaCl at 4°C for 48 hours. The external fluid

was stored in the deep freeze. On testing it showed an activity equivalent to a histamine concentration of 1.7×10^{-6} M.

Petroleum Ether Extraction: The aqueous solution was adjusted to pH 2 and shaken with 2 volumes of redistilled petroleum ether. The upper layer was removed and evaporated to dryness under reduced pressure, in an atmosphere of nitrogen. The aqueous layer was freeze-dried. Both substances were dissolved in 5 ml of Tyrode's at pH 7.5 and tested for activity on the guinea pig ileum. The organic phase had an activity equal to 1.6×10^{-7} and the aqueous phase 1.01×10^{-6} moles of histamine per liter.

Test for Histamine: A portion of the dialyzable material was taken up in *n*-butanol at pH 11. The butanol phase was passed through a CAS column and subsequently extracted with heptane. The columns were washed and treated with HCl according to the usual scheme for the determination of histamine. The acid eluates proved to be negative for histamine.

Test for Serotonin: The heptane extract was treated with 0.1 N HCl and the aqueous phase was freeze-dried. Tests on the guinea pig ileum were positive and gave a response equivalent to 3.7×10^{-7} M histamine. Additional tests for serotonin were made on a solution containing 6.83 mg/ml of active material. The solution was chemically assayed according to the method of Weissbach without first passing the material through CAS columns. The analysis showed 3.6×10^{-7} moles/liter equivalent to serotonin. Similar tests, conducted on tryptophan standards, showed that it would be necessary to increase the tryptophan concentration by a factor of 10 to get a significant change in the readings. Biological tests on tryptophan were negative.

Pharmacological Experiments

The effects of atropine, pyribenzamine, bromo-lysergic acid, and phenylbutazone were tested with respect to their blocking action against a constant dose of active material.

The results given in Table VIII, show that although bromo-lysergic acid could block a significant portion of the response only phenylbutazone was able to produce a complete blockade.

TABLES

- I. Histamine Release as a Function of Temperature
- II. Effect of Hapten Concentration on the Anaphylactic Reaction of *In Vitro* Sensitized Guinea Pig Tissue
- III. Ultracentrifugal Properties of Ragweed Preparations
- IV. Effect of Streptolysin "O" on the Atrial Action Potential of the Isolated Guinea Pig Atria at 37°C
- V. Effect of Streptolysin "O" (3×10^{-2} mg/ml) on Spontaneously Beating Isolated Guinea Pig Atria at 37°C
- VI. Effect of Streptolysin "O" on the Long Term Spontaneous Activity at the Mammalian Neuromuscular Junction at 37°C
- VII. Effect of Substrate Concentration on Velocity of Formation of Dialyzable Kinin in the Presence of Sea Urchin Toxin (SUT:[64-1], 2/3 SAS) at 37°C
- VIII. Blockade of Dialyzable Kinin with Various Agents

TABLE I
Histamine Release as a Function of Temperature

Temp. (°C)	Tissue dry wt (mg)	Moles H re- leased in 10 ml in 10 min.	Moles H re- leased per g dry wt.	Aver. total releasable H (moles)	Aver. total H per g dry weight	% of total H released in anaphylaxis
27.0	29.11	2.48×10^{-9}	0.85×10^{-7}	16.08×10^{-9}	5.52×10^{-7}	14.78%
30.0	29.93	7.09	2.36	"	5.37	38.14
32.5	22.44	2.12	0.94	"	7.16	19.24
33.5	34.54	2.12	0.61	"	4.65	28.65
36.7	26.73	8.29	3.10	"	6.01	41.90
41.1	29.76	14.40	4.83	"	5.40	62.88

1. Pigs sensitized by passive transfer of 2.0 ml of rabbit antiovalbumin (serum) containing 1.365 mg/ml precipitable antibody.
2. Atria challenged in 10 ml of solution with ovalbumin at a concentration of 0.1 mg/ml (7 x recrystallized ovalbumin).

TABLE II

Effect of Hapten Concentration on the Anaphylactic
Reaction of *In Vitro* Sensitized Guinea Pig Tissue

Prep.	Antibody 1 mg/ml for 3 hrs	Allergen mg/ml	Hapten 15 min. preceding challenge. Present during challenge mg/ml	% Blocked
RW III	"	0.15	0.00	0
	"	0.15	1.25	25
	"	0.15	1.43	28
	"	0.15	5.00	45
	"	0.15	8.00	47

TABLE III

Ultracentrifugal Properties of Ragweed Preparations

<u>Product</u>	<u>S₂₀</u>	
	Major Peak	Minor Peak
<hr/>		
<u>Allergen</u>		
I	1.06	-
III	1.84	-
IV	1.74	2.8
V	1.06	5.0
<hr/>		
<u>Hapten</u>		
I	0.62	-
III	0	-
IV	0	-
V	0.49	-
<hr/>		

TABLE IV

Effect of Streptolysin O on the Atrial Action
Potential of the Isolated Guinea Pig Atria at 37°C

Condition	Cell No.	n (no. of Recordings)	Contraction (Relative)	Action Potential (mV)	Rate of Repolarization V_{sec}^{-1}		Rate of Depolarization V_{sec}^{-1}		Repolarization Time (msec)			
					1st	2nd	3rd	max	mean	20%	80%	total
Normal	1-9	10	29.7 $\pm 0.60^*$	68.1 ± 0.38	0.936 ± 0.004	0.740 ± 0.010	0.979 ± 0.005	164 ± 1.14	36.4 ± 3.41	21.6 ± 1.14	58.0 ± 2.56	105.0 ± 2.72
Normal	9	3	31.3 ± 0.69	74.3 ± 1.85	0.932 ± 0.026	0.710 ± 0.009	0.936 ± 0.005	135 ± 3.46	34.5 ± 6.93	23.3 ± 1.67	62.0 ± 5.77	108.3 ± 3.00
Toxin**	9	9	18.9 ± 1.63	78.2 ± 2.27	1.57 ± 0.012	1.23 ± 0.012	1.51 ± 0.005	183 ± 1.07	37.6 ± 1.47	12.3 ± 1.50	38.0 ± 3.27	76.7 ± 5.40
Toxin**	9-12	12	18.5 ± 1.24	76.9 ± 1.94	1.46 ± 0.010	1.13 ± 0.010	1.39 ± 0.005	153 ± 1.27	38.6 ± 1.39	13.0 ± 1.16	40.3 ± 2.69	80.5 ± 5.00
Z Change cell 9 control to test			-39.6	+5.2	+68.5	+73.2	+61.3	+35.6	+9.0	-42.9	-71.0	-29.2
Z Change cells 1-9 + 9-12			-37.7	+12.9	+56.0	+52.7	+42.0	-6.7	+6.0	-39.8	-30.5	-23.3

* All values are mean \pm standard error

** Streptolysin "O" 1.81×10^{-2} mg/ml

Calculation made with aid of LINC, Digital Equipment Corporation, Maynard, Massachusetts.

TABLE V

Effect of Streptolysin O (3×10^{-2} mg/ml) on
Spontaneously Beating Isolated Guinea Pig Atria
at 37°C

	Rate (seconds ⁻¹)	Amplitude Relative Units
Normal	3.36 ± 0.21 *	36.3 ± 5.2
Maximal Response	2.87 ± 0.20	24.4 ± 3.5
Per Cent Change	-14.6	-32.8

* All values are mean \pm standard error

Time to maximum response (seconds) 37 ± 3.7

Time to return to control level from maximum response
(seconds) 48 ± 7.5

TABLE VI

Effect of Streptolysin "O" on the Long Term Spontaneous Activity
On the Mammalian Neuromuscular Junction at 37°C

Concentration	Control			Test			% Δ MEPP Freq.
	MEPP Freq. ± S.E. (sec ⁻¹)	MEPP Amp. ± S.E. mV	No. Fibers	MEPP Freq. ± S.E. (sec ⁻¹)	MEPP Amp. ± S.E. mV	No. Fibers	
4.35 x 10 ⁻³	11.35 ± 1.09	0.51 ± 0.06	12	10.87 ± 1.02	0.51 ± 0.05	14	- 4.3
1.49 x 10 ⁻¹	10.95 ± 0.99	0.43 ± 0.02	36	7.31 ± 1.27	0.39 ± 0.02	36	-33.2

TABLE VII

Effect of Substrate Concentration on Velocity of Formation
of Dialyzable Kiinin in the Presence of Sea Urchin Toxin
(SUT:[64-1], 2/3 SAS) at 37°C

Sea Urchin Toxin mg/ml*	Substrate mg/ml	First Order Velocity Const. $\frac{k}{\text{min}^{-1}}$	1/[S]	1/k
0.584	0.1547	0.022	6.46	44.84
"	0.1858	0.343	5.38	29.15
"	0.1981	0.469	5.05	21.32
"	0.2170	0.060	4.61	16.72
"	0.3095	0.114	3.23	8.80

* Concentrations expressed as mg protein/ml

TABLE VIII

Blockade of Dialyzable Kinin with Various Agents

Conc. of Test Compound	Blocking Agent Type	mg/ml	Initial Reaction (% of maximum)	Reaction After Blocking Agent	% Block
Dialyzable Kinin 1.21 mg/ml	Atropine	0.714	29	26.60	8.00
	*PBZ	0.114	35	26.00	25.00
	**BOL (148)	0.086	29	7.00	73.00
	"	"	21	7.50	64.50
	"	"	35	18.00	50.00
	***φBZ	1.00	30	0	100.00
Bradykinin 5.71×10^{-6} mg/ml	PBZ	0.114	50	50.00	0.00
	BOL (148)	0.086	42	0.00	100.00
Serotonin 6×10^{-7} M/L	PBZ	0.114	48	52.00	0.00
	BOL (148)	0.0857	46	0.00	100.00

* PBZ pyribenzamine

** BOL (148) dibromolysergic acid

*** φBZ phenylbutazone

SECTION II

PURIFICATION AND CHARACTERIZATION OF THE
NON-SPASMOGENIC PRINCIPLE IN CRUDE TETANUS TOXIN

I. INTRODUCTION

II. MATERIALS AND METHODS

A. *MATERIALS*

1. Parent Toxin Preparations
2. Toxoid and Antitoxin
3. Preparation of Cerebroside
4. Preparation of Ganglioside
5. Preparation of Cerebroside-Ganglioside Complex

B. *PURIFICATION METHODS*

1. Adsorption of Tetanospasmin
2. Methanol Fractionation of Tetanospasmin
3. Ammonium Sulfate Fractionation
 - a. Fraction I
 - b. Fraction II
 - c. Fraction III
 - d. Fraction IV

C. *MATERIAL YIELD OF PURIFICATION PROCEDURES*

1. Ammonium Sulfate Fractions of TD594B
(TD594B, Fractions I, II, III, and IV)
2. Cerebroside-Ganglioside Adsorption of TD594B (NSP 58-8)
3. Ammonium Sulfate Fractionation of CGC-adsorbed TD594B
(NSP 58-8 Fraction IV)

4. Cerebroside-Ganglioside Adsorption of Ammonium Sulfate-Fractionated TD661G Fraction IV (NSP 165)
5. Methanol Fractionation of TD594B (TP III)

D. *CHARACTERIZATION*

1. Biological Assays
 - a. Lethal Toxicity
 - b. Electrophysiology
2. Chemical Methods
 - a. Nitrogen Content
 - b. Light Absorption
 - c. Amino Acid Analysis
3. Physical Methods
 - a. Sedimentation Velocity
 - b. Sedimentation Equilibrium
4. Immunochemical Methods
 - a. Seroflocculation
 - b. Immunodiffusion-Immunoelectrophoresis

III. RESULTS

A. *CHEMICAL PROPERTIES*

1. Nitrogen Content of Toxin Preparations
2. Optical Density of Toxin Preparations

B. *BIOLOGICAL POTENCY*

1. Effects of Various Treatment Sequences on MEPP₅₀ Potency
 - a. Ammonium Sulfate Fractionation of TD594B (TD594B, Fractions I, II, III, and IV)
 - b. Cerebroside-Ganglioside Adsorption of TD661G Fraction IV (NSP 165)
 - c. Adsorption of TD594B Directly with CGC Complex (NSP 58-8)

d. Ammonium Sulfate Fractionation of NSP 58-8
(NSP 58-8 Fraction IV)

e. Methanol Fractionation of TD594B (TP III)

2. Effects of Various Treatment Sequences on LD₅₀ Potency

a. Ammonium Sulfate Fractionation of TD594B
(TD594B, Fractions I, II, III, and IV)

b. Cerebroside-Ganglioside Adsorption of TD661G Fraction IV
(NSP 165)

c. Adsorption of TD594B Directly with CGC Complex (NSP 58-8)

d. Ammonium Sulfate Fractionation of NSP 58-8 (NSP 58-8
Fraction IV)

e. Methanol Fractionation of TD594B (TP III)

f. Summary

C. *IMMUNOCHEMICAL ANALYSIS*

1. Seroflocculation

2. Immunoelectrophoresis

3. Immunodiffusion

D. *ULTRACENTRIFUGAL ANALYSIS*

1. Sedimentation Velocity

a. Summary

2. Molecular Weight Determination of Toxin Preparations

IV. CONCLUSIONS

V. TABLES

VI. FIGURES

INTRODUCTION

Previous studies in this laboratory (1,2) have shown that there is a factor in crude tetanus toxin which affects the peripheral neuromuscular junction by increasing the rate of spontaneous discharge of miniature end-plate potentials in preparations of isolated intercostal muscles of the mouse. This activity is unrelated to the mode of action of the classical, centrally active tetanospasmin which is attributed to blockade of spinal inhibitory synapses leading eventually to the tetanus of the extensor muscles (3).

Analysis of the mechanism of the peripheral action of the non-spasmogenic principle suggests that it lowers the resting membrane potential at the presynaptic portion of the junction and thus increases the number of units released (2). However, in spite of the increase in the numbers of acetylcholine packets liberated by the action of the toxin, the amount of acetylcholine in each packet, the quantum content, is reduced. Since the rate of replenishment of these packets is not affected by the toxin it is probable that the toxin reduces the overall degree of synthesis in the calcium-associated, voltage dependent quanta which are released by voltage changes generated across the membrane of the motor nerve terminal.

From our previous work it appeared that this non-spasmogenic principle was distinct from the lethal tetanospasmin because we could remove much of the lethal toxicity by treating the crude preparation with protagon; the specific receptor of tetanospasmin in protagon being a cerebroside-ganglioside complex (4). Adsorption with protagon left a residue having a high potency with respect to miniature end plate potentials and a greatly reduced lethal toxicity. We have also shown that the activity of the peripheral non-spasmogenic principle could be blocked by equine antitoxin; that it was thermolabile, and that the electrophysiological effect required an activation energy of 7 kilocalories.

The principal aims of the present study were to determine whether the peripheral and central effects were produced by the same substance or by different molecules present in the crude toxin and, if possible, to determine the physiochemical and immunochemical properties of the purified material. Accordingly, two independent fractionations of crude toxin were made: the first was aimed at enriching the electrophysiological potency by combined ammonium sulfate fractionation and cerebroside-ganglioside adsorption of the preparation; the second was designed to increase the lethal toxicity by the method of Pillemer (5) which is based on the purification of tetanospasmin by repeated precipitation with methanol at low temperatures.

Evidence that the two activities are probably brought about by separate molecules will be based on independent variation of the LD₅₀ and MEPP₅₀ values, immunochemical behavior and ultracentrifugal properties of the various fractions.

MATERIALS AND METHODS

MATERIALSParent Toxin Preparations

Two preparations of tetanus toxin were obtained from Dr. R. O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent. These toxin preparations were obtained from cultures of the Harvard strain of *Clostridium tetani* grown in Mueller medium and harvested by filtration through Hyflo Supercel. Preparation TD594B was a partially pure, lyophilized powder having 6.4×10^7 mouse LD₅₀/mg precipitable N, it was 42% protein and had 1740 Lf units/mg precipitable N. Preparation TD661G Fr. IV was a lyophilized product prepared directly from bacterial filtrates by removing all precipitable material up to 70% saturation with ammonium sulfate. It was 41% protein and it contained 6.09×10^4 mouse LD₅₀/mg precipitable N and 2.74×10^4 Lf units/mg precipitable N.

Toxoid and Antitoxin

Toxoid and antitoxin were also obtained from Dr. R. O. Thomson. Standard tetanus toxin MWC 3100 contained 1175 Lf units/ml and 1.28 mg precipitable N/ml. The standard Wellcome equine antitoxin FP2367 contained 2700 Lf units/ml and 10 mg precipitable N/ml.

Preparation of Cerebroside

Cerebroside was prepared by the method of Klenk and Leupold (6). Seven kilograms of fresh whole beef brain was homogenized in a Waring blender with acetone (1.3 L/kg), filtered by gravity, then re-extracted 4 times with the same volume of acetone. Then it was extracted with 5 successive volumes (1.3 L/kg) of ethyl ether in the same way.

The acetone/ether extracted brain residue was then suspended in boiling 85% ethyl alcohol (11 L/kg) and filtered while hot. On cooling, crude sphingolipids precipitated from the alcoholic filtrate were filtered off and retained. The alcoholic filtrate was used again to extract the brain residues. The pooled crude sphingolipids were suspended in ethyl ether then washed with additional ethyl ether while being gently filtered with suction on a Büchner funnel. Then the washed sphingolipids were air-dried.

The dry sphingolipids were dissolved in chloroform-methanol (3:1, V/V, 1 g/4.6 ml), with heating, and the solution filtered while warm. The precipitate that formed on standing at room temperature for 24 hours was filtered with suction, redissolved, and then reprecipitated in the same manner. The resulting precipitate was filtered with suction then dissolved in hot glacial acetic acid (10 ml/g, V/W) and left to stand overnight for precipitation. The next day the precipitate was

filtered, washed with acetone (20 ml/g, V/W) and desiccated for 4 hours under reduced pressure. The resulting crude cerebrosides were dissolved in pyridine (40 ml/g, V/W) and the solution was percolated through 1.5 cm(I:D.) columns containing alumina prepared according to the method of Brockmann (1 g alumina/8 ml solution). The eluate was reduced to one-eighth of its original volume under reduced pressure, brought back to its original volume with acetone, cooled to 4°C, then held there at 4°C for one hour. The resulting precipitate was filtered, then desiccated as before. The total yield of pure cerebroside was 10 g or 1.3 g/kg fresh whole brain.

Preparation of Ganglioside

Ganglioside was prepared by the method of van Heyningen and Miller (7). Fresh whole beef brain, 3.5 kg, was homogenized with acetone (3 L/kg), filtered, re-extracted again, then air-dried. The dry brain was extracted with chloroform-methanol (1:2, V/V, 2 L/kg) at 60°C with continuous stirring for 30 minutes. The extract was filtered off and the residue re-extracted again with the same volume of solvent. The pooled extracts were left at 4°C overnight. The resulting precipitate was filtered off and dissolved in chloroform-methanol (4:1, V/V, 50 g/L).

Activated silicic acid (Mallinckrodt 100 mesh) was added (1 g/g dry weight) to the solution and the mixture was stirred for 2 minutes. The suspension was then centrifuged in a Model PR-2 International centrifuge fixed-angle head Type 845, at 3,000 rpm for 15 minutes then washed 3 successive times with chloroform-methanol (3:1, V/V), 8 ml per gram silicic acid. The ganglioside was then eluted twice with 8 ml methanol per gram silicic acid. The methanol eluate was then mixed with 2 volumes of chloroform and dialyzed against 2 changes of 10 volumes of distilled water for 48 hours. The upper phase was concentrated on a rotary evaporator to 20 ml for each kilogram of original fresh brain then lyophilized. The material was redissolved in a small volume of water, centrifuged at 3,000 rpm for 30 minutes to remove residual silicic acid, then re-lyophilized. The resulting yield was 2.1 grams or 0.6 g/kg fresh whole brain.

Preparation of Cerebroside-Ganglioside Complex

A mixture consisting of 3.6 g of cerebroside and 1.2 g of ganglioside was dissolved in 130 ml of warm MeOH-CHCl₃ (1:1). The solvent was removed under vacuum over a steam bath, and the residue dried for 60 minutes. The precipitate was then suspended in 50 ml of boiling water and shaken for 5 minutes. A sufficient quantity of water was added to bring the final volume of the cerebroside-ganglioside (CGC) suspension to 120 ml.

PURIFICATION METHODS

Adsorption of Tetanospasmin

The adsorption procedure of tetanospasmin with cerebroside-ganglioside complex was similar to that previously described for

adsorption with protagon (1). Toxin preparations were prepared for adsorption by dissolving one gram of lyophilized powder in 80 ml of 0.1 M phosphate buffer (pH 7.0). The resulting solution was treated with 20 ml of the CGC suspension and after vigorous shaking, aliquots were delivered into plastic cups and centrifuged at 23,500 rpm (48,000 x g) for 20 minutes in a Spinco Model L preparative ultracentrifuge. The original solution was extracted 6 times in this manner. The supernatant was dialyzed against 1/60 M PO_4 pH 7.0 and lyophilized. The dried product is referred to as a "non-spasmogenic preparation (NSP)."

Methanol Fractionation of Tetanospasmin

Purification of tetanospasmin was based on differences of solubility of proteins in low dielectric media in the presence of methanol as the organic precipitating agent under controlled conditions of pH, ionic strength, and temperature. A detailed description of this fractionation follows; and it is shown in the form of a flow sheet in Figure 1.

One volume of tetanus toxin solution was dissolved in 0.1M NaCl, chilled to 1°C, adjusted to pH 5.15 with ice-cold HAC-NaAc 0.01M (pH 4), and immediately transferred to a -5°C bath. To this mixture a sufficient quantity of absolute methanol, measured at -5°C and chilled to -20°C, was slowly added with constant stirring to make the solution overall 40 per cent methanol. The precipitate was allowed to settle out of solution for 24 hours at -5°C. The precipitated material was then removed by centrifugation at -5°C, dissolved in ice-cold 0.15 M NaAc (pH 6.8) and diluted with H_2O to 0.095 M. The pH was then adjusted to 5.5 with HAC-NaAc 0.01 M and at -4°C a methanol-water mixture was added to bring the solution up to 15% methanol and an ionic strength of 0.025. This solution was allowed to settle out 24 hours at -4°C. The precipitate was then removed at -4°C, dissolved in 0.15 M NaAc, and reprecipitated at pH 4.0 in the presence of 15% methanol, and ionic strength of 0.025. The mixture was maintained for 24 hours at -4°C and the precipitate was removed by centrifugation at -4°C, dissolved in 0.075 M NaAc, pH 6.8, and lyophilized.

Ammonium Sulfate Fractionation

The fractionation of parent toxin TD5943 and other preparations was based on their relative solubilities in three concentrations of ammonium sulfate equivalent to 35, 70, and 100 per cent saturation at room temperature. The toxin preparations were fractionated according to the general scheme used by Murata *et al.* (8) to yield four fractions. A detailed description of the fractionation follows and is shown as a flow sheet in Figure 2.

Fraction I: Fraction I was separated as a free suspension of particles at room temperature in the presence of 35 per cent saturated ammonium sulfate. The toxin preparations were dissolved in 0.1 M phosphate buffer and then made up to 35 per cent saturation by the addition of saturated ammonium sulfate. The pH of the suspension was maintained

at 7.0 with 5 N NaOH. After the suspension had settled, the residue was centrifuged down with the aid of an International centrifuge swinging bucket head, Type 269, at 2,500 rpm for 30 minutes at 13°C. The residue was then dissolved in water and made up to 35 per cent saturation for a second time by the addition of saturated ammonium sulfate solution. The second precipitate was taken up in water and a small amount of insoluble material was allowed to settle out overnight. After the residue was removed by centrifugation, Fraction I, contained in the supernate, was dialyzed and dried from the frozen state.

Fraction II: The supernate in 35 per cent saturated ammonium sulfate which remained after the removal of Fraction I was cooled to 4°C and the pH was lowered to 3.2 by the addition of 6 N HCl. Three hours after the precipitate had formed it was centrifuged down at 2°C and then dissolved in distilled water. Enough saturated ammonium sulfate solution was added to the solution to bring the salt saturation to 35 per cent, and then the acid treatment was repeated. The residue was taken up in distilled water, dialyzed, and lyophilized.

Fraction III: The first supernate remaining after removal of Fraction II was neutralized with 5 N NaOH in the cold and brought to 70 per cent saturation with saturated ammonium sulfate solution at room temperature. The precipitate was allowed to coacervate at room temperature for 1 day. The insoluble material was removed by filtration and the filtrate reprecipitated in the presence of 70 per cent saturated ammonium sulfate pH 7.0. The residue was recovered by centrifugation at 29°C, dialyzed, and then lyophilized.

Fraction IV: The fourth fraction was separated from the filtrate remaining after the removal of Fraction III by 70 per cent ammonium sulfate. This fraction was allowed to settle and then was centrifuged. The residue was dissolved in distilled water, dialyzed, and then reprecipitated in saturated ammonium sulfate. The second residue was dissolved in water, dialyzed, and lyophilized.

As a final step each fraction as described above was resuspended in 1/60 M phosphate-buffered saline and then dialyzed against 1/60 M phosphate alone. The first step of dialysis, which removed most of the ammonium sulfate, involved 3 to 4 changes of 1% NaCl in 1/60 M phosphate. This was followed by dialysis against two changes of 1/60 M phosphate buffer at pH 7.2.

MATERIAL YIELD OF PURIFICATION PROCEDURES

Ammonium Sulfate Fractions of TD594B (Fractions I, II, III, and IV)

Treatment of 948 mg of TD594B yielded a total of 452 mg of material which was distributed among the four fractions in the following way: there was 83 mg of Fraction I, 194 mg of Fraction II, 94 mg of Fraction III, and 80 mg of Fraction IV. The total yield was 47.7

per cent of the weight of starting material and Fraction IV represented slightly over 8.4 per cent of the total.

Cerebroside-Ganglioside Adsorption of TD594B (NSP 58-8)

Adsorption of 1.0 gm of TD594B successively with 20 ml of CGC suspension yielded 229 mg of NSP 58-8, or 23% of the starting material.

Ammonium Sulfate Fractionation of CGC-adsorbed TD594B (NSP 58-8 Fr. IV)

Processing of 160 mg of NSP 58-8 for Fraction IV resulted in a yield of 42 mg of NSP 58-8, Fraction IV, or 26% of the starting material.

Cerebroside-Ganglioside Adsorption of Ammonium Sulfate Fractionated TD661G Fr. IV (NSP 165)

The material balance of Fraction IV of TD661G could not be obtained because the ammonium sulfate fractionation was made directly on liquid bacterial filtrates rather than on the lyophilized powder. The large scale fractionation by Dr. R. O. Thomson produced 2.64 g of Fraction IV of which 1.0 g was used for adsorption by cerebroside-ganglioside. The yield of NSP 165 from that amount of Fraction IV was 478.6 mg, 48%, or approximately double the proportion of material yielded by treating the crude toxin with the adsorbent.

Methanol Fractionation of TD594B (TP III)

The lyophilized product contains a large proportion of salt since dialysis is omitted in methanol fractionation. The total yield of TP III from 100 mg of TD594B is expressed as 21 per cent of the total precipitable Nitrogen present in the starting material.

CHARACTERIZATION

Biological Assays

Each preparation was assayed for lethal toxicity to estimate the tetanospasmin content, and for electrophysiological potency to measure the content of the non-spasmogenic principle. Both of these procedures have been described in detail previously (1, 2).

Lethal Toxicity: Determinations of LD₅₀ were made by injecting 20 g Swiss-Webster mice with various concentrations of a given preparation, the intervals being so chosen as to permit the construction of a complete dose-mortality curve for each preparation. The samples were diluted with 0.15 M phosphate buffer pH 7 which contained 0.1% W/V of gelatin and the preparations were injected in doses of 0.5 ml given intramuscularly into the hind legs of the test animals. Four mice were used for each concentration of the material under assay, and seven days were allotted for the test.

Electrophysiology: Electrophysiological assays were performed on isolated hemithoraces (2) obtained from 20 g Swiss-Webster mice. The preparations were mounted in a perspex clamp then placed in a muscle bath and maintained with Liley's solution at 37°C. Intracellular potentials were measured with KCl-filled microelectrodes that were connected by an Ag-AgCl bridge to the input of a cathode-follower circuit. The potentials were displayed on one beam of a Tektronix 502 oscilloscope and photographed with a Grass oscilloscope camera. Potentials were recorded from at least twelve cells before and after the introduction of a test solution. The results were considered acceptable only if the frequency remained stable, the amplitude at least 250 μ V and the resting membrane potential exceeded -60 mV. The average resting frequency was $5.35 \pm 0.29 \text{ sec}^{-1}$ at 37°C. The observations were reduced by plotting the mean percentile increments of test to control frequency obtained in at least 5 experiments against the final concentration of toxin present in the bath. The range of response is limited to a 50% increase above control frequency and the preparations were quantitatively bioassayed by the concentration required to produce one-half the limiting response. The midpoint of the reaction curve, the MEPP₅₀, was then used as an index analogous to the LD₅₀ to evaluate the electrophysiological potency.

Chemical Methods

Nitrogen Content: Nitrogen was estimated according to the Kjeldahl-Nessler micromethod as modified by Lanni *et al.* (9) and Feigen *et al.* (10). The nitrogen content was determined directly on 1 ml aliquots of toxin solution as well as on precipitates formed in the presence of ice cold 5% (W/V) trichloroacetic acid.

Light Absorption: All absorbance measurements were made on a Beckman DU spectrophotometer in the range 230 to 400 m μ . The optical densities of the toxin solutions were measured by standard methods in quartz cuvettes having a light path of 1 cm.

Amino Acid Analysis: The analyses were performed by Dr. Allen Grossberg of Roswell Park on a Technicon Automatic Amino Acid Analyzer using a single column system with accelerated (6 1/2 hr) gradient elution according to a modification of the method described by Piez and Morris (11). The samples used were hydrolyzed in 6 N HCl at 108°C in quadruplicate: at 22, 44, 66, and 89 hours, respectively.

Physical Methods

Sedimentation Velocity: These measurements were made with the aid of a Spinco Model E analytical ultracentrifuge at 52,640 rpm (201,366 x g) in 0.15 M phosphate buffer, pH 7.0, at 20°C, with toxin solutions at concentrations of 0.33 to 3.36 mg precipitable N/ml. Toxin separation experiments were made with a single sector fixed-partition cell under the same conditions.

Sedimentation Equilibrium: Molecular weights were determined from coupled synthetic boundary and sedimentation equilibrium runs at 12,590 rpm (11,520 x g), the same toxin solutions being used for both determinations. Calculation of molecular weights from the concentration and concentration gradient data were made with the use of apparent specific volumes derived from amino acid composition data (12).

Immunochemical Methods

Seroflocculation: The serological potency of the preparations was estimated by Ramon titration (13). This involved the determination of the optimal proportions point in a set of antigen-antibody mixtures containing 0.2 ml aliquots of various dilutions of standard equine antitoxin (Wellcome Research Laboratories FP2367, 2700 Lf units/ml) and 0.2 ml of a constant concentration of antigen. The optimal proportions point was determined by finding the fastest flocculation time in the measurement of flocculation times at 41°C and by interpolation from graphs relating flocculation time to antibody concentration.

Immunodiffusion-Immunelectrophoresis: The number of antigenic determinants of the toxin preparations was analyzed by the modified Ouchterlony immunodiffusion technique and by the immunelectrophoretic method of Grabar and Williams (14). Immunodiffusion patterns formed by material diffusing from filter-paper discs on 1% agar were allowed to develop over 24-48 hours. Immunelectrophoresis of toxin preparations was carried out in 0.05M barbital buffer-1% agar (pH 8.2) at 25 milliamps for 2.5 hours. Antitoxin was then added and allowed to diffuse for 24-48 hours. All samples were stained with amido Black 10B.

RESULTS

Since we were interested initially in the isolation of a substance other than tetanospasmin we considered it necessary to determine first if the electrophysiological activity could be distributed independently of the lethal toxicity by ammonium sulfate fractionation; then, if found possible, to adsorb the most electrophysiological active fraction with CGC to determine whether a higher degree of electrophysiological potency could be obtained in this way.

The general plan was first to fractionate crude tetanus toxin with ammonium sulfate and treat the most active fraction with CGC, then to reverse the procedure by adsorbing crude toxin directly with CGC and fractionating the non-adsorbed daughter preparation with ammonium sulfate in order to compare which sequence was most efficient in purifying the electrophysiological activity. Also, to demonstrate that lethal toxicity could be concentrated independently of electrophysiological activity, crude toxin was fractionated with methanol at low temperatures and the resulting product TP III was compared with parent TD594B and the other fractions

in terms of LD₅₀, MEPP₅₀, immunochemical, and ultracentrifugal properties.

CHEMICAL PROPERTIES

Nitrogen Content of Toxin Preparations

The results of the nitrogen analyses which are presented in Table I show that parent material, TD594B, is approximately 9 per cent nitrogen based on dry weight. Among the ammonium sulfate precipitated fractions the greatest nitrogen content was found in the acid-precipitated Fraction II, and the lowest in Fraction I; there was no essential difference between Fraction III and IV, each of which had 6.8 to 6.6 per cent total nitrogen. The ratio of the precipitable to total nitrogen (P/T) was approximately 90 per cent in Fraction I, II, and III, while in Fraction IV the P/T ratio was about 71 per cent. Following the treatment of parent TD594B with the CGC complex, the total nitrogen was seen to increase from 9 per cent in the parent material to 11 per cent in NSP 58-8; the precipitable nitrogen, however, was essentially unchanged so that the ratio of precipitable to total nitrogen was decreased by the treatment. NSP 58-8 Fraction IV showed considerable reduction both in the total nitrogen, which was 3 per cent as compared to 11 per cent in the parent NSP preparation, and in the amount of nitrogen precipitable by TCA. The ratio of precipitable to total nitrogen was reduced from 65 per cent in the parent preparation to 46 per cent in Fraction IV.

Preparation TD661G Fraction IV was 9 per cent nitrogen by weight; the precipitable nitrogen content was 7 per cent, giving a ratio of precipitable to total nitrogen of 75 per cent. After cerebroside-ganglioside adsorption, the daughter preparation, NSP 165, showed a slightly greater total, and a somewhat lower precipitable, content of nitrogen than its parent material; thus the P/T ratio was reduced from 75 to 67 per cent.

Preparation TP III was only 0.9 per cent nitrogen by weight owing to methanol fractionation and direct lyophilization; however, the precipitable nitrogen content was 0.8 per cent to yield a P/T ratio of 89 per cent.

Optical Density of Toxin Preparations

Absorbance measurements at 280 mμ were used to characterize the toxin preparations and to follow those fractionation procedures that resulted in increased optical densities (Table I).

Determinations of the absorption spectrum were made in the range of 200 to 400 mμ on solutions of TD594B and NSP 58-8, containing 0.68 and 0.072 mg/ml precipitable nitrogen, made up in 0.15 M phosphate buffer at pH 7.0. Additional curves were run on solutions of TD661G and NSP 165. As can be seen from Figure 3, both TD594B and NSP 58-8 show a peak at 260 mμ, the effect of CGC treatment apparently being to intensify this peak per unit precipitable N. This observation is

consistent with the finding that both the total and precipitable nitrogen values are increased by CGC treatment. As is evident from Figure 4 the CGC treatment of Fraction IV TD661G produces a small change in the spectrophotometric properties of NSP 165. It therefore appears that treatment of the crude preparation with the adsorbent removes a large amount of inert material so as to concentrate certain species of proteins having different biological properties but a uniform proportion of components absorbing in the ultraviolet.

BIOLOGICAL POTENCY

Effects of Various Treatment Sequences on MEPP₅₀ Potency

Ammonium Sulfate Fractionation of TD594B (Fractions I, II, III, and IV): The effects of ammonium sulfate fractionation alone on parent TD594B are shown in Figure 5. Fraction I, precipitated at 35 per cent saturated ammonium sulfate pH 7 was the least active at 2×10^{-6} mg/ml. Fraction II, acid-precipitated at pH 3.2, 0.35 saturated ammonium sulfate, and Fraction III, precipitated at 0.75 saturated ammonium sulfate, were about as potent as parent TD594B. The potency of Fraction IV was more than twice that of the parent, 7.0×10^{-10} versus 1.6×10^{-9} mg/ml.

Cerebroside-Ganglioside Adsorption of TD661G Fraction IV (NSP 165): Since Fraction IV of TD594B was shown to be the most electrophysiologically active, a large scale fractionation of Fraction IV at 70-100 per cent saturated ammonium sulfate directly from bacterial filtrates was made to produce TD661G Fraction IV. This was then adsorbed with CGC to yield NSP 165. Figure 6 shows that CGC treatment of Fraction IV increased its MEPP₅₀ potency from 1×10^{-7} to 4.5×10^{-12} mg/ml and reduced the heterogeneity of the preparation. NSP 165 had the highest MEPP₅₀ potency of all the preparations produced.

Adsorption of TD594B Directly with CGC Complex (NSP 58-8): The comparison of TD594B and its post-adsorption material, NSP 58-8, in Figure 7 shows that the primary effect of this treatment is to reduce the heterogeneity of the preparation while increasing the MEPP₅₀ potency from 2×10^{-9} to 5.5×10^{-10} mg/ml.

Ammonium Sulfate Fractionation of NSP 58-8 (NSP 58-8 Fr IV): Fractionation of NSP 58-8 to produce Fraction IV further increases the MEPP₅₀ potency from 5.5×10^{-10} to 3×10^{-11} mg/ml (Figure 8).

Methanol Fractionation of TD594B (TP III): The elimination of electrophysiological activity by methanol fractionation of TD594B is shown in Figure 9 which exhibits the extremes of MEPP₅₀ potency of preparations derived from a common parent. Methanol fractionation of TD594B has reduced its MEPP₅₀ potency from 1.6×10^{-9} to 1.4×10^{-3} mg/ml. Compared to NSP 58-8 Fraction IV with a MEPP₅₀ potency of 3×10^{-11} this is a difference of 8 orders of magnitude in electrophysiological activity.

Effects of Various Treatment Sequences on LD₅₀ Potency

The effects of combined ammonium sulfate fractionation-CGC adsorption sequences and of the methanol fractionation of crude toxin on LD₅₀ potency are shown in Table II which compares MEPP₅₀ and LD₅₀ potencies on the basis of milligrams precipitable nitrogen. It can be seen that the two potencies are independently variable and that the MEPP₅₀/LD₅₀ ratio is a good index of the effectiveness of the separation procedure.

Ammonium Sulfate Fractionation of TD594B (Fractions I, II, III and IV): Fractionation of TD594B is shown to decrease progressively the lethal toxicity of the starting material. The lethal toxicity is reduced from 6.44×10^7 LD₅₀/mg precipitable N in the starting material to 4.26×10^6 LD₅₀/mg precipitable N in Fraction IV. At the same time, however, this treatment increases the MEPP₅₀ potency from 7.73×10^9 to 3.08×10^{10} /mg precipitable N with a resulting increase of the efficiency ratio from 1.20×10^2 to 7.23×10^3 .

CGC Adsorption of TD661G Fraction IV (NSP 165): Further adsorption with CGC of Fraction IV (produced by ammonium sulfate fractionation) resulted in a further decrease in LD₅₀ potency. TD661G Fraction IV, though it initially had a lower LD₅₀ than TD594B Fraction IV as it had a different parent, showed a reduction in potency from 6.09×10^4 to 3.3×10^2 LD₅₀/mg precipitable N in NSP 165. The resulting efficiency ratio of 1.23×10^{10} was 7 orders of magnitude higher than the starting ratio of 2.46×10^3 in TD661G Fraction IV.

Adsorption of TD594B Directly with CGC Complex (NSP 58-8): The abrupt increase in the efficiency ratio of NSP 58-8 over its parent by 4 orders of magnitude, from 1.20×10^2 to 1.94×10^6 , is due mainly to the large decrease in lethal toxicity from 6.44×10^7 to 2.34×10^4 LD₅₀/mg precipitable N.

Ammonium Sulfate Fractionation of NSP 58-8 (Fraction IV): Fractionation of NSP 58-8 resulted in the decrease of LD₅₀ from 2.34×10^4 to 1.45×10^2 LD₅₀/mg precipitable N in NSP 58-8 Fraction IV. Since there was an almost equivalent increase in MEPP₅₀ from 4.53×10^{10} to 1.44×10^{12} MEPP₅₀/mg precipitable N, the efficiency ratio increased from 1.94×10^6 to 9.93×10^9 .

Methanol Fractionation of TD594B (TP III): Enrichment of lethal toxicity is seen in the methanol treatment of parent TD594B. There is an increase from 6.44×10^7 to 3.99×10^8 LD₅₀/mg precipitable N in TP III. Coupled with this increase in LD₅₀ there is a far greater decrease in magnitude of the MEPP₅₀ potency, from 7.73×10^9 to 8.92×10^4 MEPP₅₀/mg precipitable N. This results in an efficiency ratio of 2.24×10^{-4} for TP III, demonstrating undeniably that methanol fractionation concentrates lethal toxicity independently of electrophysiological activity.

Summary: Comparison of the effects of various treatment sequences on the toxin preparations by parallel electrophysiological activity and lethal toxicity assays indicates both potencies can be selectively concentrated. Combined ammonium sulfate fractionation and CGC adsorption of crude toxin in either sequence; either by first fractionating the parent and then adsorbing the most active fraction, or by adsorbing the parent first and then fractionating, appears to produce the most electrophysiologically active preparations. NSP 165 and NSP 58-8 Fraction IV, having almost identical LD₅₀, MEPP₅₀, and efficiency ratio values, demonstrate that both sequences of treatment are virtually equal in effectiveness for the purification of the electrophysiological factor.

Methanol fractionation of crude toxin produces a material having slightly increased lethal toxicity but greatly decreased electrophysiological activity. This result demonstrates that lethal toxicity can be concentrated independently of electrophysiological activity.

IMMUNOCHEMICAL ANALYSIS

The effects of various treatment sequences on the antigenic composition of the toxin preparations were assessed by standard seroflocculation, immunoelectrophoresis, and immunodiffusion techniques.

Seroflocculation

The results of the Ramon titrations performed on various preparations of toxin are exhibited as time-flocculation curves. It is generally accepted that the time of flocculation varies with the concentration of the reagents but that the position of the optimal proportions point in a set is constant, since it depends on the combining ratio of antibody to antigen. Figure 10 shows that the Ramon optimal proportions titration had the usual form and that the Lf content was progressively increased with ammonium sulfate fractionation of TD594B. The range of optimal flocculation times (K_f) is seen to be 10 to 20 minutes between parent TD594B and its fractions, parent TD594B having a K_f of 20 minutes. Figure 11 shows that a further increase in Lf content is produced by CGC adsorption of TD661G ammonium sulfate Fraction IV and that a slight reduction of 10 minutes in the K_f occurred in the daughter preparation NSP 165.

When parent TD594B is directly adsorbed with CGC to yield NSP 58-8 the flocculation potency is increased; however, there is also a great increase in K_f from 20 to 460 minutes (Figure 12). Further ammonium sulfate fractionation of NSP 58-8 to NSP 58-8 Fraction IV, in turn, while increasing the flocculative potency, reduces the K_f from 460 to approximately 90 minutes. That CGC treatment of parent crude toxin before fractionation should decrease the velocity of flocculation while both CGC adsorption after fractionation and fractionation of the CGC adsorbed parent should increase the velocity of flocculation, is not entirely clear. However, all three procedures increase the Lf content of the starting material and this is summarized in Table III. It is

seen that the serological potency of parent TD594B is increased from 1.74×10^3 to 2.87×10^4 Lf units/mg precipitable N in TD594B Fraction IV by ammonium sulfate fractionation. CGC adsorption of TD594B to yield NSP 58-8 results in a slight improvement of Lf content to 6.02×10^3 Lf units/mg precipitable N, while ammonium sulfate fractionation of NSP 58-8 to NSP 58-8 Fraction IV results in further increase of Lf content to 3.04×10^4 Lf units/mg precipitable N. CGC adsorption of TD661G Fraction IV results in a slight increase of Lf content from 2.74×10^4 to 3.51×10^4 Lf units/mg precipitable N. It is apparent that serological potency increases abruptly with ammonium sulfate fractionation while CGC adsorption has a definite but slight effect.

Immuno-electrophoresis

Immuno-electrophoretic analysis showed that parent TD594B was extremely heterogenous antigenically. It had a total of 20 antigenic determinants against equine tetanus antitoxin. The lower well in Figure 13, slide 123, shows that the antigenic determinants of TD594B are distributed in two major and three minor bands of precipitin arcs. Ammonium sulfate fractionation of TD594B results in the gradual diminution and elimination of antigenic determinants so that in Fraction IV, there remains a 12 line system.

Direct adsorption of TD594B with CGC results in a similar reduction in the intensity and number of lines, as seen in Figure 13, slide 126. However, the distribution of the antigen determinants in NSP 58-8 is slightly different from that of TD594B Fraction IV and there also appears to be more diffuse negative material in the former preparation. Further fractionation of NSP 58-8 with ammonium sulfate to NSP 58-8 Fraction IV, as shown in Figure 13, slide 127, results in even greater diminution and elimination of lines so that essentially a 2 arc system remains.

The antigenic characterization of TD661G Fraction IV and its CGC-adsorbed daughter, NSP 165, is not directly comparable to the other preparations because they were produced from a different parent material. However, they both appear similar to their counterparts, TD594B Fraction IV and NSP 58-8 Fraction IV, respectively, in their content of major antigens. Figure 14, slide 121, shows that TD661G Fraction IV and TD594B Fraction IV appear very similar and although TD661G Fraction IV seems to have 4 fewer lines than the 12 in TD594B Fraction IV, both share the major antigenic determinants. Further adsorption of TD661G Fraction IV with CGC appears to have removed the antigenic determinant directly over the origin and diminished some of minor negative components (Figure 14, slide 107).

In comparing NSP 165 to NSP 58-8 Fraction IV, (Figure 14, Slide 130) the most electrophysiologically active fractions that possessed nearly identical LD_{50} , $MEPP_{50}$, and efficiency ratio values, it is evident that they share only the major positive and the major negative

component in each pattern. This finding suggests that the biological effects are associated with these two antigenic determinants.

Methanol fractionation of TD594B to produce TP III; the preparation that possessed the highest lethal toxicity and low electrophysiological activity, resulted in the virtual elimination of all but one major arc. Figure 14, slide 138, compares TD594B to TP III and it can be seen that TP III has essentially one major positive component plus, at best, trace negative components.

Since NSP 58-8 Fraction IV and NSP 165, the most electrophysiologically potent preparations, share but 2 principal arcs, one positive and one negative, it is possible to identify the MEPP stimulating antigen by eliminating the one associated with the lethal factor. Moreover, since the single major positive arc in TP III corresponds to that given by crystalline tetanospasmin, which is known to migrate as a β -globulin (15), this suggests that the major negative component in NSP 58-8 Fraction IV and NSP 165 is the most probable locus of the MEPP factor.

Immunodiffusion

Double-diffusion antigenic analysis, though lacking the resolving power of immunoelectrophoresis, provided preliminary characterization of the molecular nature of the antigenic determinants. Figure 15, slide 99, compares TD594B, NSP 58-8 and NSP 58-8 Fraction IV when tested against equine antitoxin in the center well. It can be seen that the heavier molecules present in parent TD594B are very much reduced in NSP 58-8, and that further fractionation of NSP 58-8 removes virtually all of the heavy material. Figure 15, slide 95, matches TD594B with TD661G Fraction IV and it shows that there is no heavy material in TD661G Fraction IV that is comparable to that contained in TD594B. Further adsorption of TD661G Fraction IV with CGC to yield NSP 165, as seen in Figure 16, slide 88, does not seem to change the amount of the lighter material present in both preparations.

Immunodiffusion of methanol-fractionated TP III reveals that it consists almost entirely of heavy material and that it shows little, if any, identity with the light material in TD661G Fraction IV (Figure 16, slide 107).

It appears that combined ammonium sulfate fractionation and CGC adsorption eliminates the heavier moieties in crude toxin while methanol fractionation of TD594B, on the other hand, serves to concentrate these heavy moieties. This suggests, therefore, that electrophysiological activity is associated with the lighter fractions and that the centrally acting lethal factor is concentrated in the heavier molecules.

ULTRACENTRIFUGAL ANALYSIS

Sedimentation velocity analyses, performed on each of the toxin preparations to determine the number of molecular species present,

provided closer examination of the nature of the heavy and light molecules observed in the immunodiffusion tests.

Parent TD594B was found to have 3 major components: 7S, 4S, and 14S. Figure 17 shows that the 7S component was the major moiety and that 4S and 14S were minor components. The Svedberg coefficients of crude tetanus toxin and other preparations analyzed exhibited the usual dependence on concentrations; i.e., slightly higher sedimentation rate of proteins when in more dilute solutions. Owing to the scarcity of material it was not always possible to make determinations at more than one concentration; therefore, the lineage of sedimenting peaks was identified as belonging to a given cluster of Svedberg values. Table IV summarizes the results of the sedimentation velocity analyses. It can be seen that ammonium sulfate fractionation alone of TD594B results in the gradual elimination of the heavier components so that in Fraction IV TD594B there remains but a single major 3-4S component (Figure 17).

Direct CGC treatment of TD594B to yield NSP 58-8 results in similar elimination of the heavier major 7S component present prior to adsorption but there remains a heavy component which was possibly unadsorbed or was added by complex formation (Figure 18). There also remains a good deal of very light material which obscures the homogeneity of the 4S peak. Further fractionation of NSP 58-8 results in a slight improvement in homogeneity of the 4S peak present in NSP 58-8 Fraction IV. This is shown in Figure 19.

That ammonium sulfate fractionation of parent toxin followed by CGC adsorption is a better method of preparing an ultracentrifugally purer product is shown in Figure 20 which compares TD661G Fraction IV and its CGC adsorbed daughter NSP-165. TD661G Fraction IV is seen to have one major 4S peak, and another 9-10S peak is added by CGC adsorption owing to complex formation. The fact that this 9-10S peak added by CGC adsorption is considered unimportant was shown by trap-out experiments on NSP 165 utilizing the fixed-partition cell. Figure 21 shows the leading 9-10S peak being trapped out from the NSP 165 mother preparation. Upon re-analysis of the mother preparation by sedimentation velocity, as illustrated in Figure 21, it is seen that the 9-10S peak has been effectively removed from NSP 165. Immunoelectrophoresis of this material, as seen in the upper well in Figure 22, slide 131, when compared to intact NSP 165, shows that the antigenic composition remains essentially unaltered when the 9-10S complex peak is removed.

It appears that the main effect of CGC adsorption on TD661G Fraction IV, as seen again in Figure 20 which compares TD661G Fraction IV to NSP 165, is to reduce the height of the trailing shoulder at the meniscus and increase the homogeneity of the 4S peak.

The significance of the very light material which was seen in all of the previous sedimentation figures as the broad heterogenous boundary that remained close to the meniscus is revealed in the lower series of patterns of Figure 23 which shows the sedimentation pattern of

formalinized tetanus toxoid. The fact that tetanus toxoid on immunodiffusion (Figure 22, slide 120) gives but one major and one trace minor heavy arc indicates that the very light material is non-antigenic and therefore not associated with either electrophysiological or lethal activity.

Methanol fractionation of TD594B to yield TP III results in a product that apparently possesses only one major 7S peak (Figure 23). This information plus evidence from the previously mentioned immunodiffusion analysis of TP III, which revealed only the presence of heavy moieties, indicates that the antigenic determinants of TP III are found in the 7S fraction. It is significant that TP III, which has the highest lethal activity of the toxin preparations, and tetanus toxoid, which is inactive, are both 7S moieties.

Summary: By sedimentation velocity analysis it can readily be seen that combined ammonium sulfate fractionation and CGC adsorption procedures concentrate the 4S moieties while they eliminate the 7S and 13-14S components. On the other hand, methanol fractionation concentrates the 7S molecules and eliminates the 4S and 13-14 S components present in parent TD594B. From corroborative evidence gained from MEPP₅₀, LD₅₀, and immunochemical analyses, it is evident the MEPP factor is concentrated in the 4S moiety and that the lethal factor is concentrated in the 7S toxin molecules.

Molecular Weight Determination of Toxin Preparations

While sedimentation velocity studies provided information about the association of MEPP factor with 4S molecules and the concentration of lethal factor in 7S molecules, sedimentation equilibrium analyses made possible the determination of the approximate molecular weights of these moieties. These analyses were limited to TD661G Fraction IV and TP III, since these were the least heterogeneous of the preparations having high MEPP₅₀ and high LD₅₀ activity, respectively.

The molecular weight values were calculated by the Archibald method from the measurement of concentration distributions during the approach to sedimentation equilibrium with use of the partial specific volumes of the macromolecules. The partial specific volume of 0.743 cc/g for TD661G Fraction IV was determined directly from its amino acid composition. The results of the amino acid analysis along with the calculation of the partial specific volume is shown in Table V. The apparent specific volumes of the constituent amino acids are those tabulated by Cohn and Edsall and the method of calculation is that described by Schachman (16). Due to insufficient material the partial specific volume of 0.749 cc/g used for TP III was that determined for crystalline tetanospasmin by Largier (17).

Using these values TD661G Fraction IV was found to have a molecular weight of 59,495 while TP III had a molecular weight of 172,000. These molecular weights are in the same range as the

crystalline tetanus toxin monomer and dimer which have molecular weights of 68,000 and 146,000, respectively. As a check on the molecular weight value obtained by sedimentation equilibrium, a direct calculation of molecular weight from amino acid composition by the method of Dunn *et al.* (18) was performed on TD661G Fraction IV. These results are shown in Table VI. The average molecular weight calculated on this basis was found to be 57,074. This is in close agreement with the figure of 59,495 found by sedimentation equilibrium.

CONCLUSIONS

We have shown that the two physiological activities in crude tetanus toxin are properties of different molecules present in the mixture. A partial separation has been effected and the physical properties of the two factors have been determined:

1. Purification of the MEPP factor by the combined treatment of the crude preparation with ammonium sulfate and cerebroside-ganglioside enriches the electrophysiological potency 200-fold and decreases the lethal toxicity half a million-fold.
2. Partial purification of the tetanospasmin by methanol precipitation decreases the MEPP factor 90,000-fold and increases the lethal toxicity 6-fold.
3. The two factors can be separated immuno-electrophoretically.
4. The activity of the MEPP factor is concentrated with the 4S group of proteins and that of the lethal factor with the 7S proteins.
5. Estimates of the molecular weight show that the lethal factor has a value of 176,000 and that the MEPP factor has a value of about 59,000.

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SECTION II

TABLES

- I. Nitrogen Analyses and Optical Densities at 278 m μ of Toxin Preparations.
- II. Biological Potency
- III. Seroflocculation
- IV. Distribution of Molecular Species in Toxin Preparations
- V. Partial Specific Volume of TD661G Fraction IV
- VI. Molecular Weight of TD661G Fraction IV

SECTION II

FIGURES

1. Methanol Purification for TP III
2. Ammonium Sulfate Fractionation Procedure for Tetanus Toxin and NSP
3. The Effect of Cerebroside-Ganglioside Treatment of Crude Tetanus Toxin on Light Absorption
4. The Effect of Cerebroside-Ganglioside Treatment of TD661G Fraction IV on Light Absorption
5. MEPP Frequency of Parent Toxin TD594B and Ammonium Sulfate Fractions I, II, III, and IV
6. MEPP Frequency of TD661G Fraction IV and NSP 165
7. MEPP Frequency of TD594B and NSP 58-8
8. MEPP Frequency of NSP 58-8 and NSP 58-8 Fraction IV
9. MEPP Frequency of Tetanus Toxin TD594B, NSP 58-8 Fraction IV and TP III
10. Seroflocculation of Parent Toxin TD594B and Ammonium Sulfate Fractions II, III, and IV
11. Seroflocculation of TD661G Fraction IV and NSP 165
12. Seroflocculation of NSP 58-8 and NSP 58-8 Fraction IV
13. Immuno-electrophoresis of TD594B, TD594B Fraction IV, NSP 58-8, and NSP 58-8 Fraction IV
14. Immuno-electrophoresis of TD661G Fraction IV, TD594B Fraction IV, NSP 165, NSP 58-8 Fraction IV, TD594B, and TP III
15. Immunodiffusion of TD594B, NSP 58-8, NSP 58-8 Fraction IV, and TD661G Fraction IV
16. Immunodiffusion of NSP 165, TD661G Fraction IV, TP III, and TD594B
17. Sedimentation Velocity Patterns of TD594B and TD594B Fraction IV

18. Sedimentation Velocity Patterns of TD594B and NSP 58-8
19. Sedimentation Velocity Patterns of NSP 58-8 and NSP 58-8 Fraction IV
20. Sedimentation Velocity Patterns of TD661G Fraction IV and NSP 165.
21. Fixed-partition Cell Separation of NSP 165 and Sedimentation Velocity Re-analysis
22. Immunelectrophoresis of NSP 165 and Separated NSP 165; Immunodiffusion of TD594B and Toxoid.
23. Sedimentation Velocity Patterns of TP III and Toxoid.

TABLE I - TABLE VI

TABLE I

Nitrogen Analyses and Optical Densities at 278 mμ
of Toxin Preparations

Material	Mg Total N/mg dry material	Mg Pptable N/ Mg dry material	P/T as%	OD ₂₇₈ / Mg dry Prep.	OD ₂₇₈ / Mg Pptble N
<u>TD594B</u>					
Crude	0.088	0.068	77.25	1.064	15.65
Fr. I	0.042	0.039	91.96	0.346	8.87
Fr. II	0.095	0.087	91.56	1.291	18.45
Fr. III	0.068	0.061	89.88	0.763	12.51
Fr. IV	0.066	0.047	70.58	0.833	17.72
<u>NSP 58-8</u>					
Parent	0.111	0.072	65.42	1.718	23.86
Fr. IV	0.030	0.014	45.95	0.399	28.60
<u>TD661G</u>					
Fr. IV	0.088	0.066	75.42	1.211	18.35
NSP 165	0.093	0.062	66.66	1.323	21.34
<u>TP III</u>					
	0.009	0.008	89.00	0.150	18.75

TABLE II
BIOLOGICAL POTENCY

Material	LD ₅₀ /mg Precipitable N	MEPP ₅₀ /mg Precipitable N	MEPP ₅₀ /LD ₅₀
Crude TD594B	6.44×10^7	7.73×10^9	1.20×10^2
Fraction I	6.44×10^4	1.26×10^7	1.96×10^2
A Fraction II	2.31×10^7	5.04×10^9	2.18×10^2
Fraction III	4.06×10^7	7.93×10^9	1.95×10^2
Fraction IV	4.26×10^6	3.08×10^{10}	7.23×10^3
B NSP 58-8	2.34×10^4	4.53×10^{10}	1.94×10^6
Fraction IV	1.45×10^2	1.44×10^{12}	9.93×10^9
C TD661G Fr. IV	6.09×10^4	1.50×10^8	2.46×10^3
NSP 165	3.30×10^2	4.06×10^{12}	1.23×10^{10}
D TP III	3.99×10^8	8.92×10^4	2.24×10^{-4}

TABLE III
SEROFLOCCULATION

Material	Lf units/mg Precipitable N
Crude TD594B	1.74×10^3
Fraction II	1.49×10^3
Fraction III	2.74×10^3
Fraction IV	2.74×10^3
Fraction IV	2.87×10^4
NSP 58-8	6.02×10^3
NSP 58-8 Fr. IV	3.04×10^4
TD661G Fr. IV	2.74×10^4
NSP 165	3.51×10^4

TABLE IV

Distribution of Molecular Species in Toxin Preparations

Svedberg Units

Material	3-4	6-7	9-10	13-14	MEPP ₅₀ ±LD ₅₀
Toxoid	-	+	-	-	Inactive
TD594B	+	+	-	±	1.2×10^2
Fraction II	+	+	-	±	2.0×10^2
Fraction III	+	+	-	±	2.0×10^2
Fraction IV	+	-	-	-	7.0×10^3
NSP 58-8	+	-	-	±	2.0×10^6
Fraction IV	+	-	-	-	1.1×10^{10}
TD661G Fr. IV	+	-	-	-	2.5×10^3
NSP 165	+	-	+	-	1.0×10^{10}
TP III	-	+	-	-	2.24×10^{-4}

TABLE V

PARTIAL SPECIFIC VOLUME OF TD661G FR. IV

Amino Acid	$\frac{\mu \text{ moles}}{\text{mg protein}}$	$\frac{W_i}{(\% \text{ by Weight of residue})}$	$\frac{V_i}{(\text{Specific Volume of residue})}$	$\frac{V_i W_i}{(\% \text{ By Volume of residue})}$
Arg	0.215	2.93	0.70	2.05
Asp	1.510	14.84	0.60	8.90
Glu	1.120	12.42	0.67	8.32
Gly	0.802	3.88	0.64	2.48
Hist	0.076	0.82	0.67	0.55
Isoleu	0.805	7.85	0.90	7.07
Leu	0.662	6.47	0.90	5.82
Lys	1.280	14.15	0.83	11.60
Meth	0.129	1.47	0.75	1.10
Phen	0.286	3.62	0.77	2.79
Thr	0.730	6.38	0.70	4.47
Val	1.000	8.54	0.86	7.34
Ser	0.525	3.97	0.63	2.50
Pro	0.460	3.88	0.76	2.95
Ala	0.790	4.83	0.74	3.57
1/2 Cys	0.060	0.52	0.63	0.32
Tyr	0.242	3.36	0.71	2.39

$$\Sigma W_i = 99.93$$

$$\Sigma W_i V_i = 74.22$$

$$\Sigma W_i V_i / \Sigma W_i = V_p = 0.743 \text{ cc/g}$$

TABLE VI

MOLECULAR WEIGHT OF TD661G FR. IV

Amino Acid	% Amino Acid in Toxin	Min. Mol. Wt. Based on One Amino Acid residue/mole ^a	Min. Amino Acid Residue/mole ^b	Min. Mol. Wt. Based on N Amino Acid Residue/mole ^c
		(A)	(B)	(C)
Arginine	2.93	5,946	10	59,460
Aspartic Acid	14.84	890	64	56,960
Glutamic Acid	12.42	1,185	48	56,880
Glycine	3.88	1,935	29	56,115
Histidine	0.82	18,921	3	56,763
Isoleucine	7.85	1,671	34	56,814
Leucine	6.47	2,027	28	56,756
Lysine	14.15	1,097	52	57,044
Methionine	1.47	10,150	6	60,900
Phenylalanine	3.62	4,563	12	54,756
Threonine	6.38	1,867	30	56,010
Valine	8.54	1,372	41	56,252
Serine	3.97	2,647	21	55,587
Proline	3.88	2,967	19	56,373
Alanine	4.83	1,845	31	57,195
Half Cystine	0.52	23,105	2	46,210 ^f
Tyrosine	3.36	5,392	11	59,312

^a = (Mol. Wt. Amino Acid/% of Amino Acid in Toxin) x 100

^b = Calculated on Basis of Histidine as 3

^c = (A) x (B)

^f = Omitted in Calculating Average

57,074 = Av.

FIGURE 1 - FIGURE 2

Purification Procedures

For TP III, TD594B, and NSP 58-8

Methanol Purification for TP III

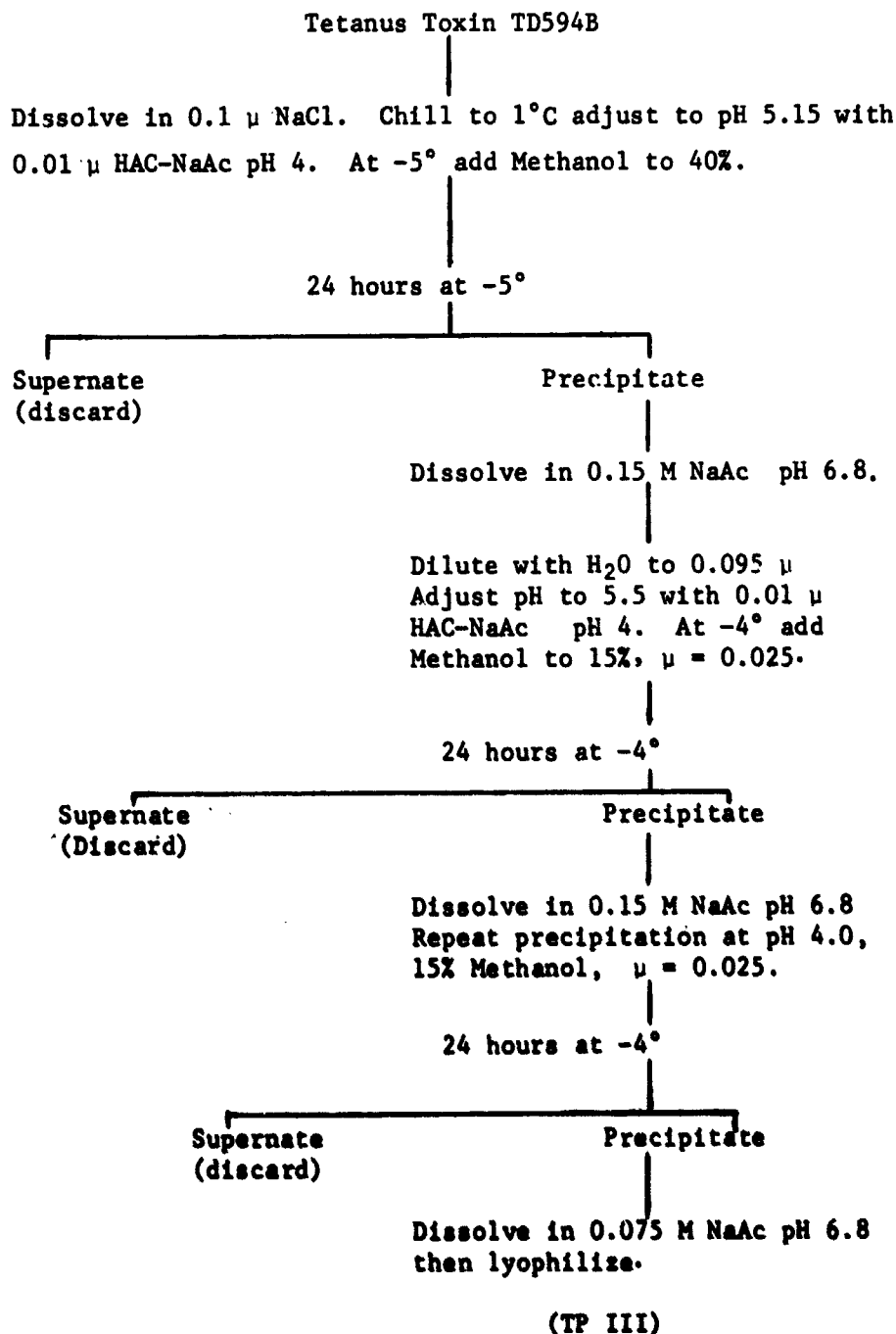


FIGURE 2

AMMONIUM SULFATE FRACTIONATION PROCEDURE FOR TETANUS TOXIN AND NSP

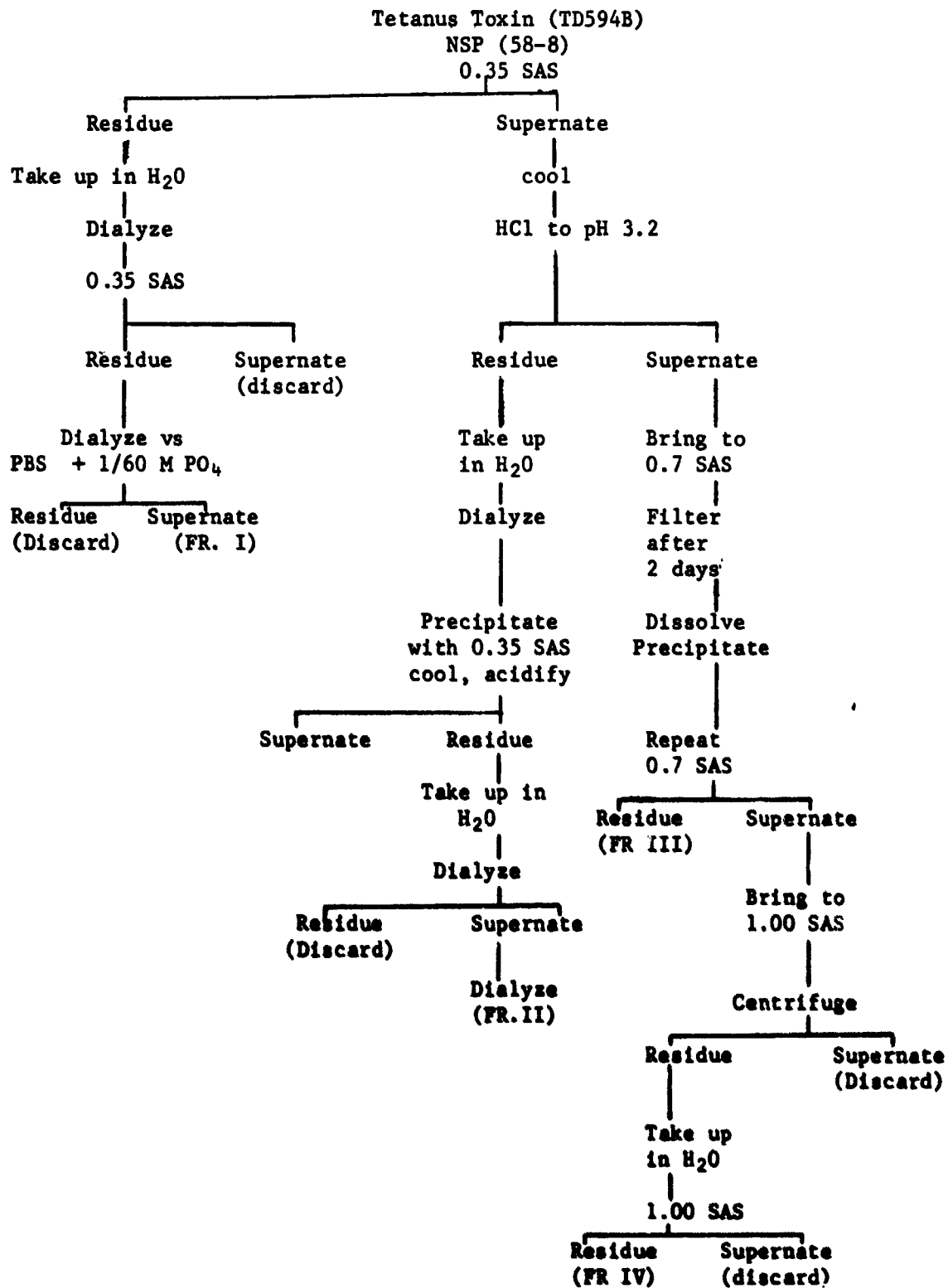
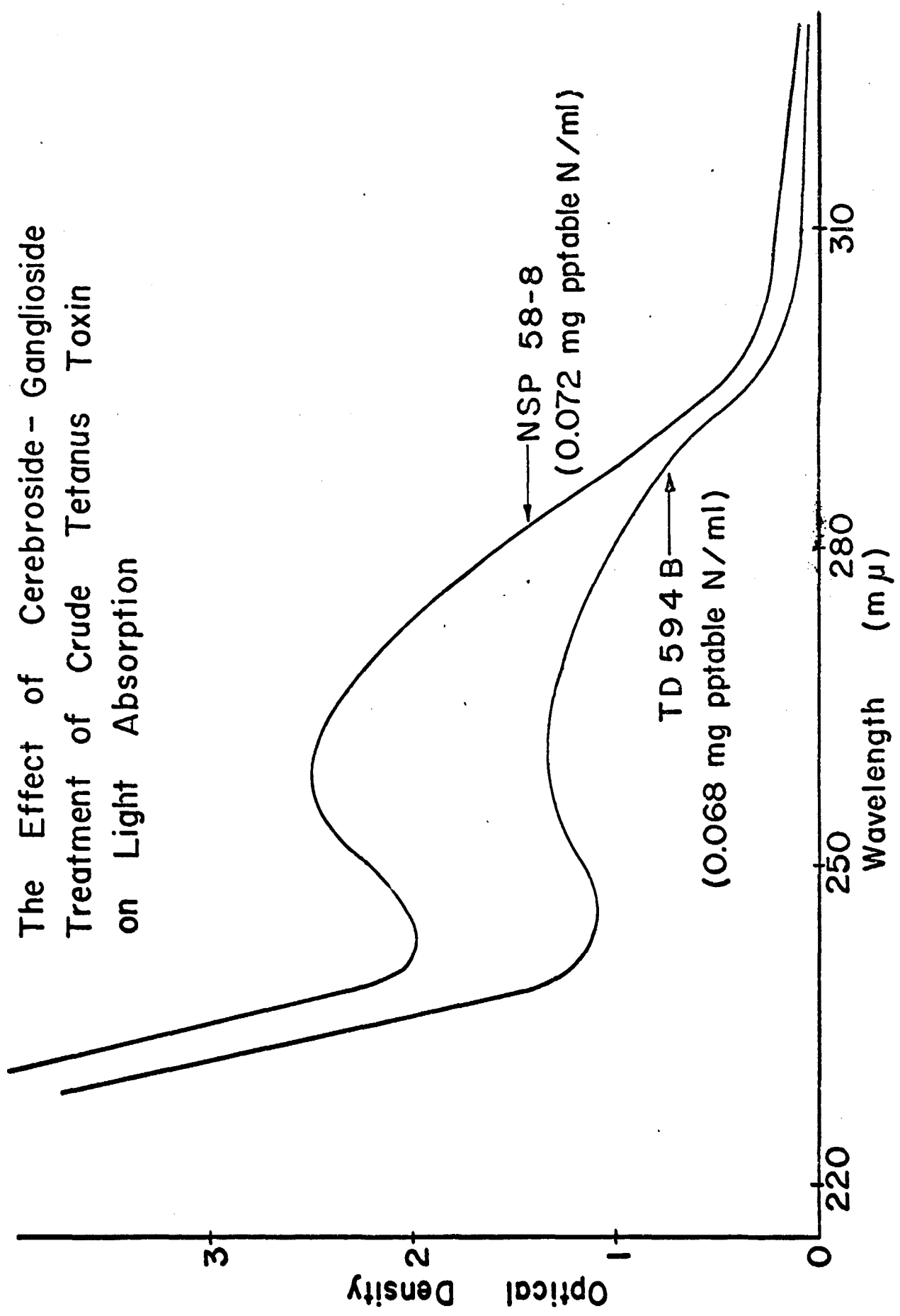


FIGURE 3 - FIGURE 4

Light Absorption Curves

The Effect of Cerebroside - Ganglioside
Treatment of Crude Tetanus Toxin
on Light Absorption



The Effect of Cerebroside - Ganglioside
Treatment of Ammonium Sulfate
Fraction IV on Light Absorption

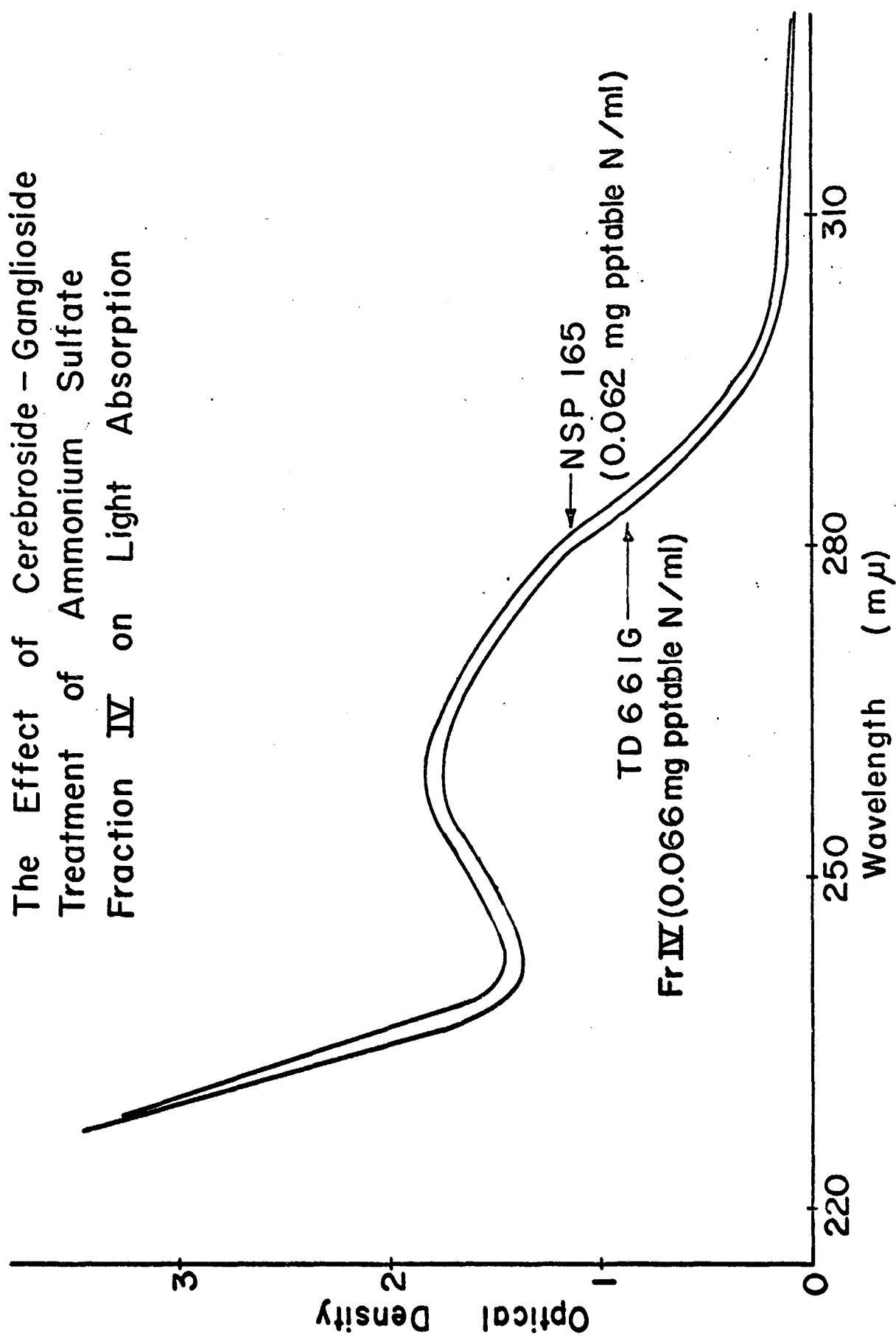
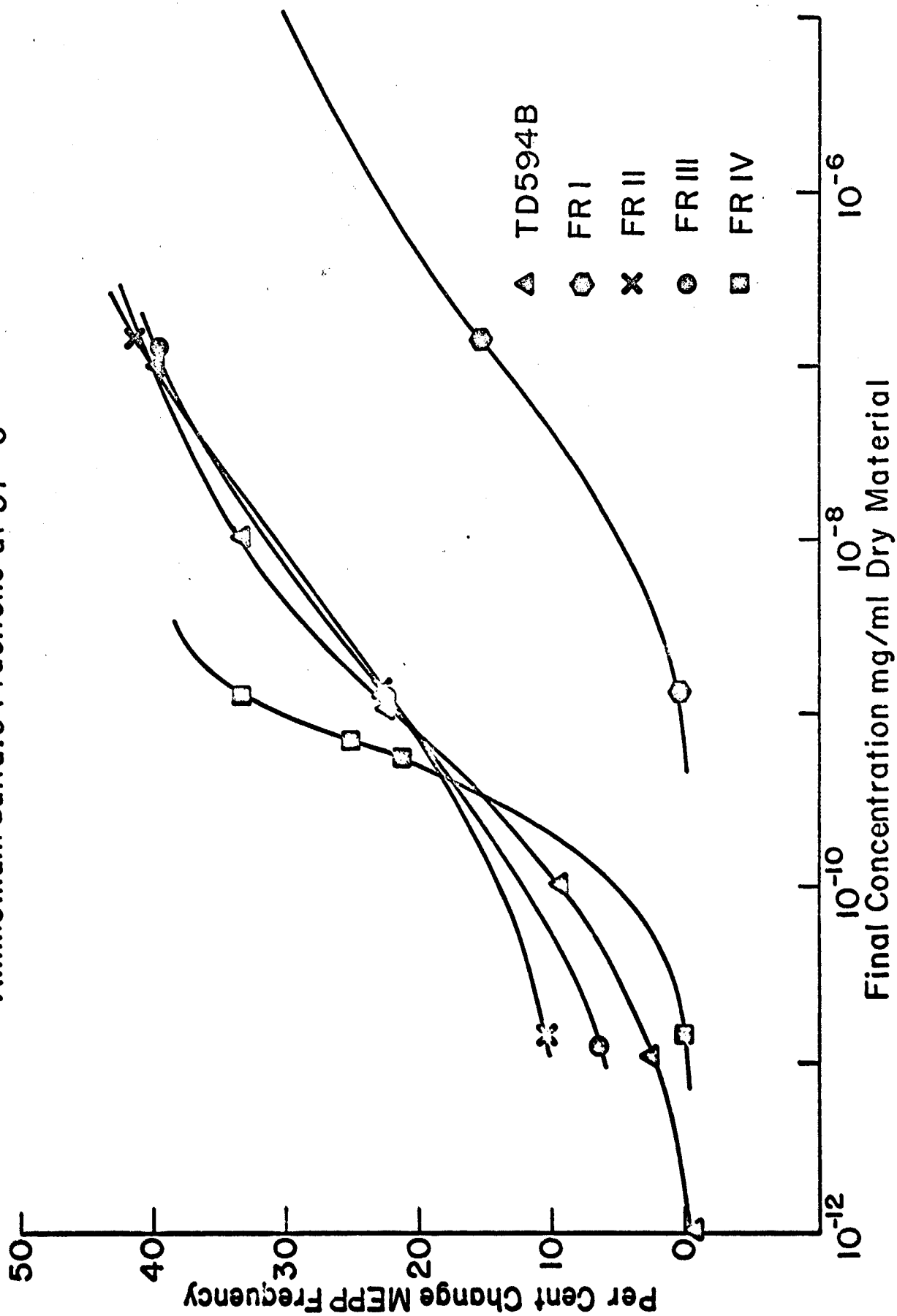


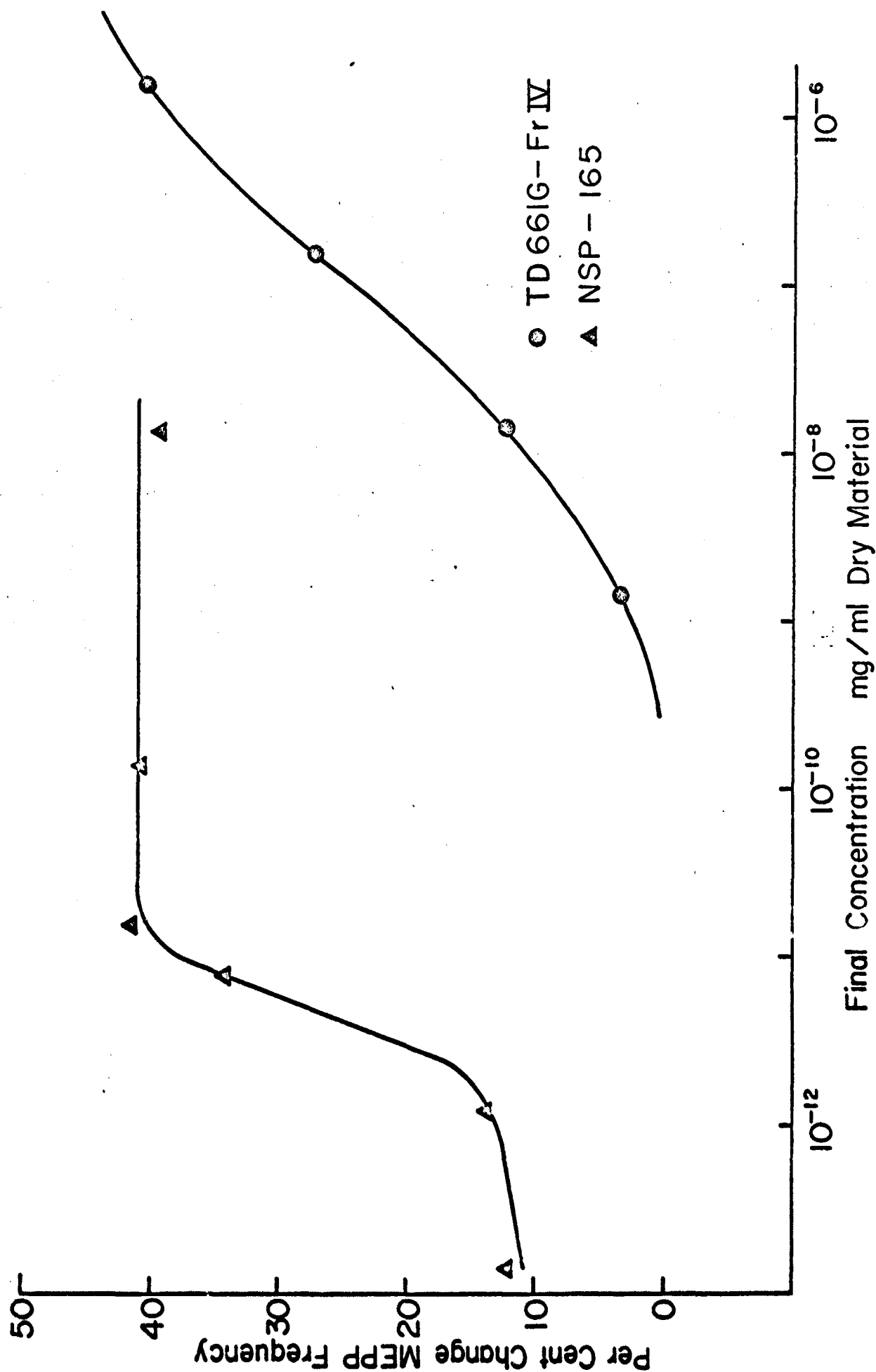
FIGURE 5 - FIGURE 9

MEPP Frequency Analysis

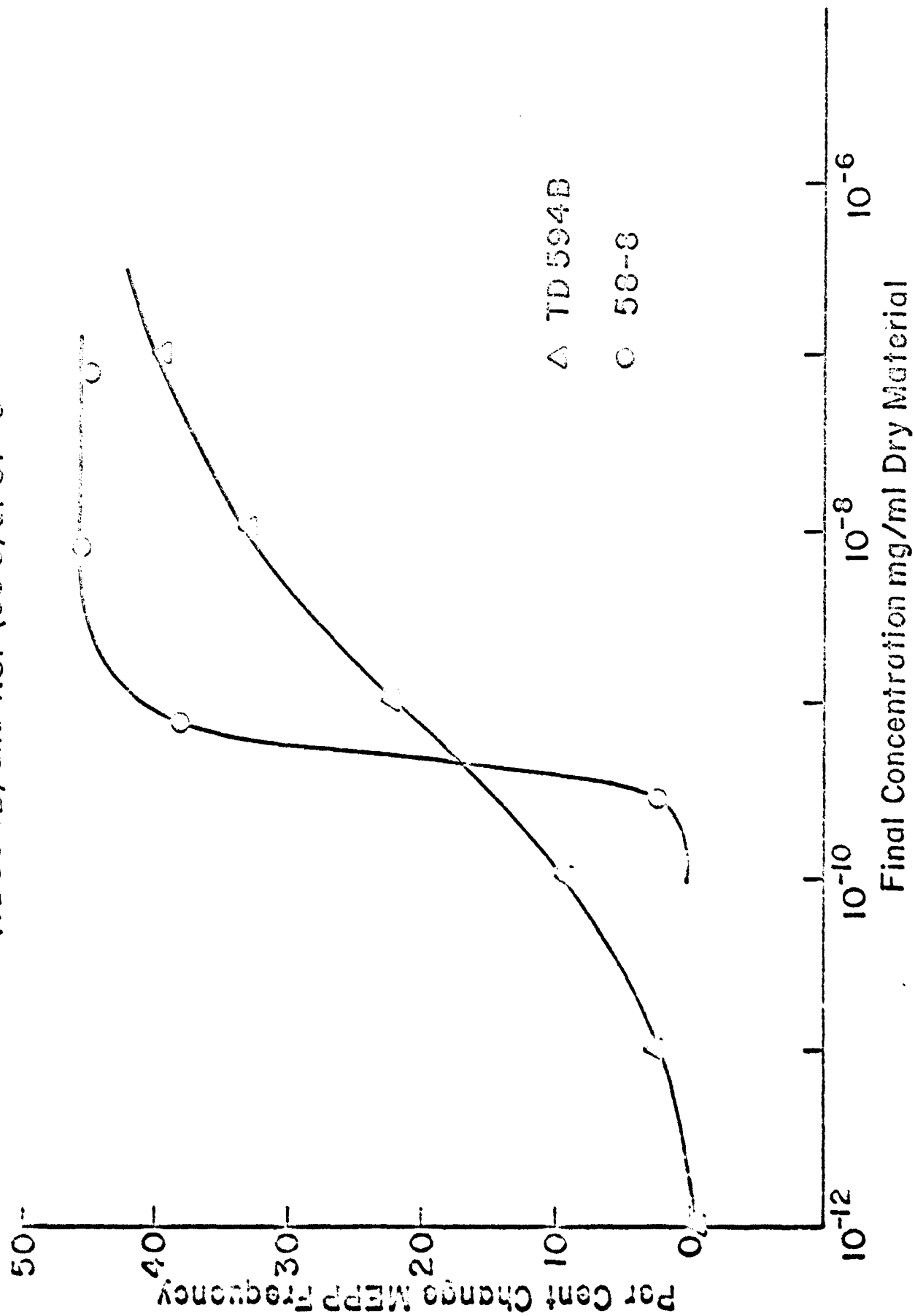
MEPP Frequency of Parent Toxin TD594B and Ammonium Sulfate Fractions at 37° C



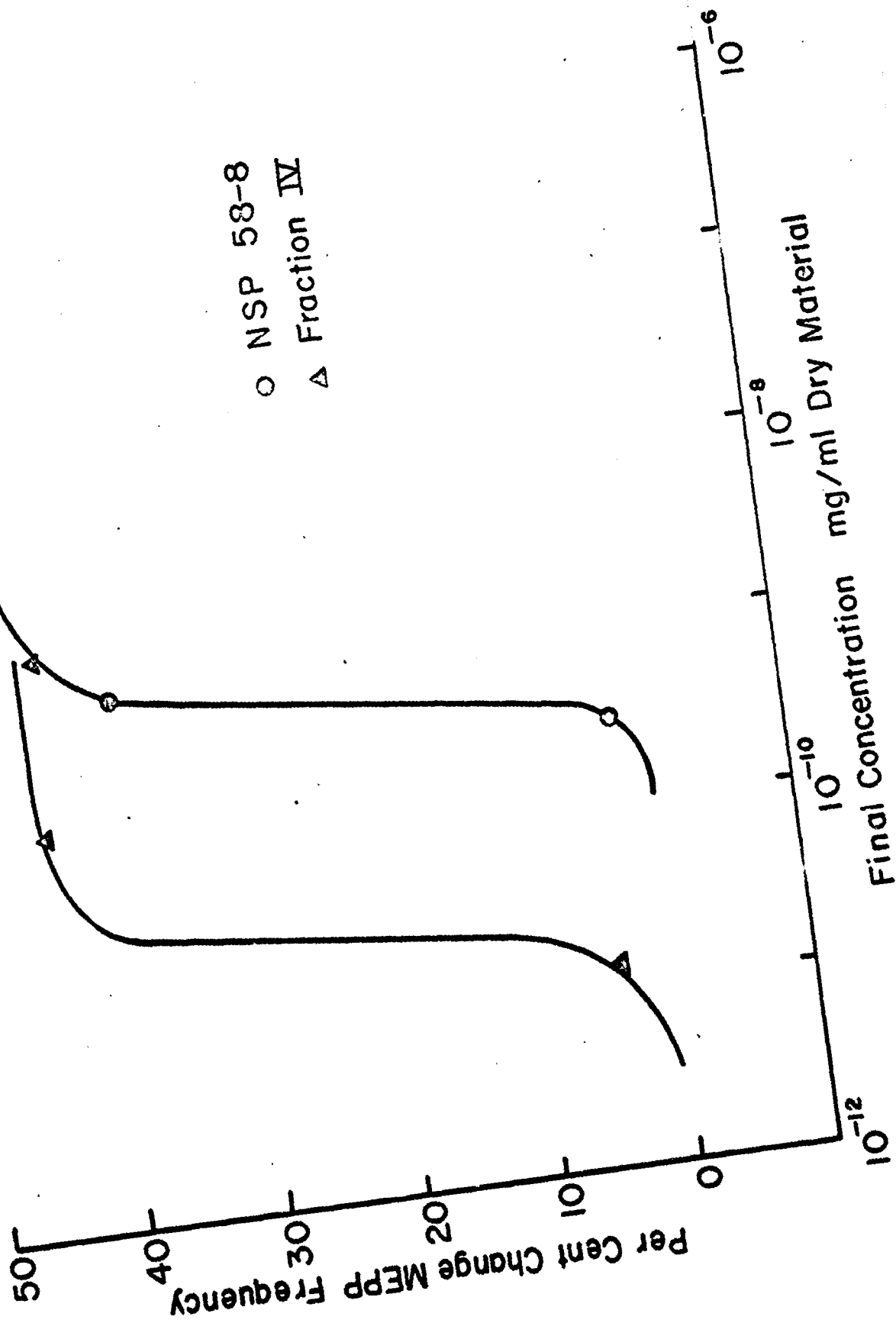
MEPP Frequency of Ammonium Sulfate Fraction IV (TD66IG) and the Non Spasmogenic Principle (I65)



MEPP Frequency of Toluene Toxin
(TD594B) and NSP (58-8) at 37°C



MEPP Frequency of
NSP 58-8 and Ammonium Sulfate Fraction IV at 37°C



MEPP Frequency of Tetanus Toxin (TD594B), NSP 58-8 FRIV, and TP III at 37°C

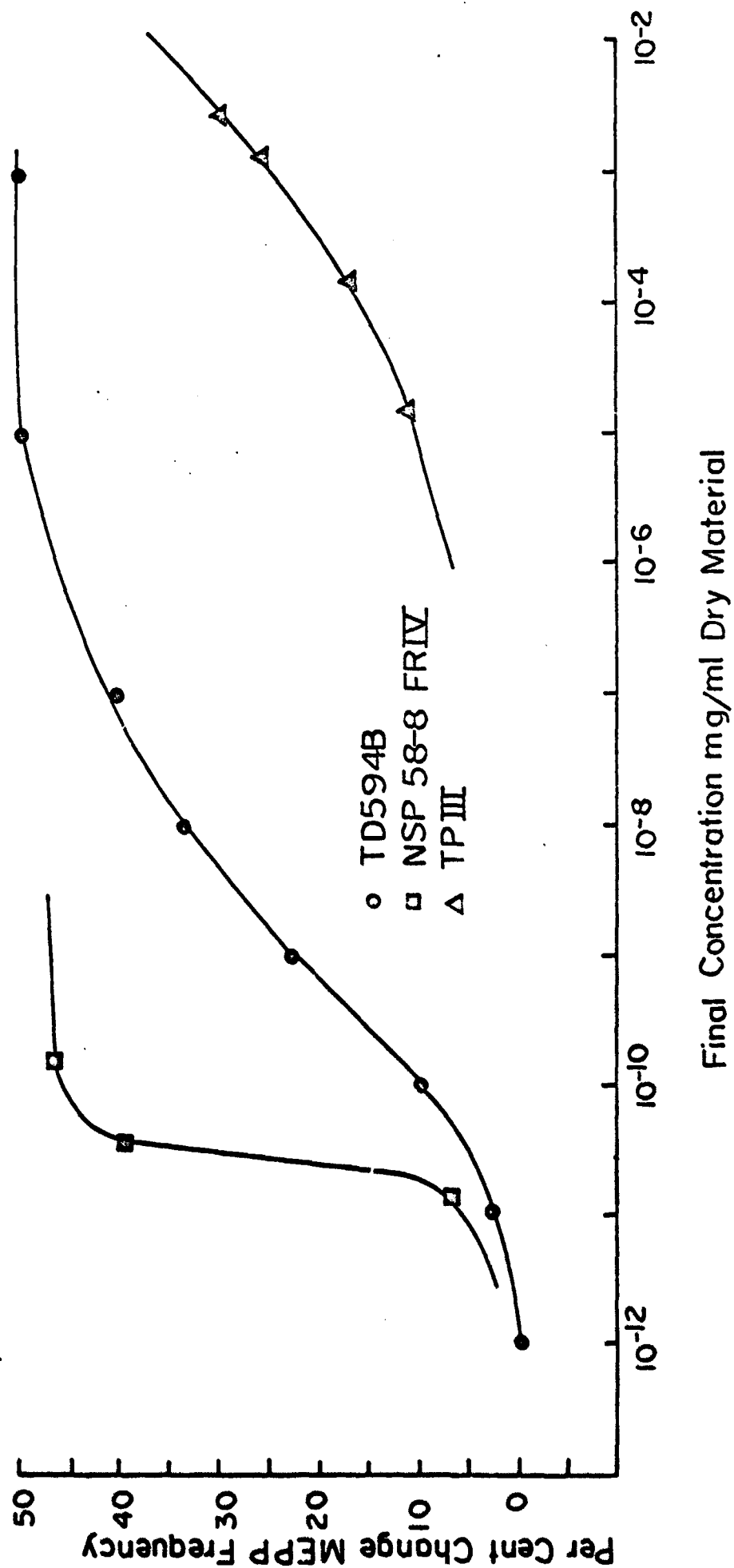
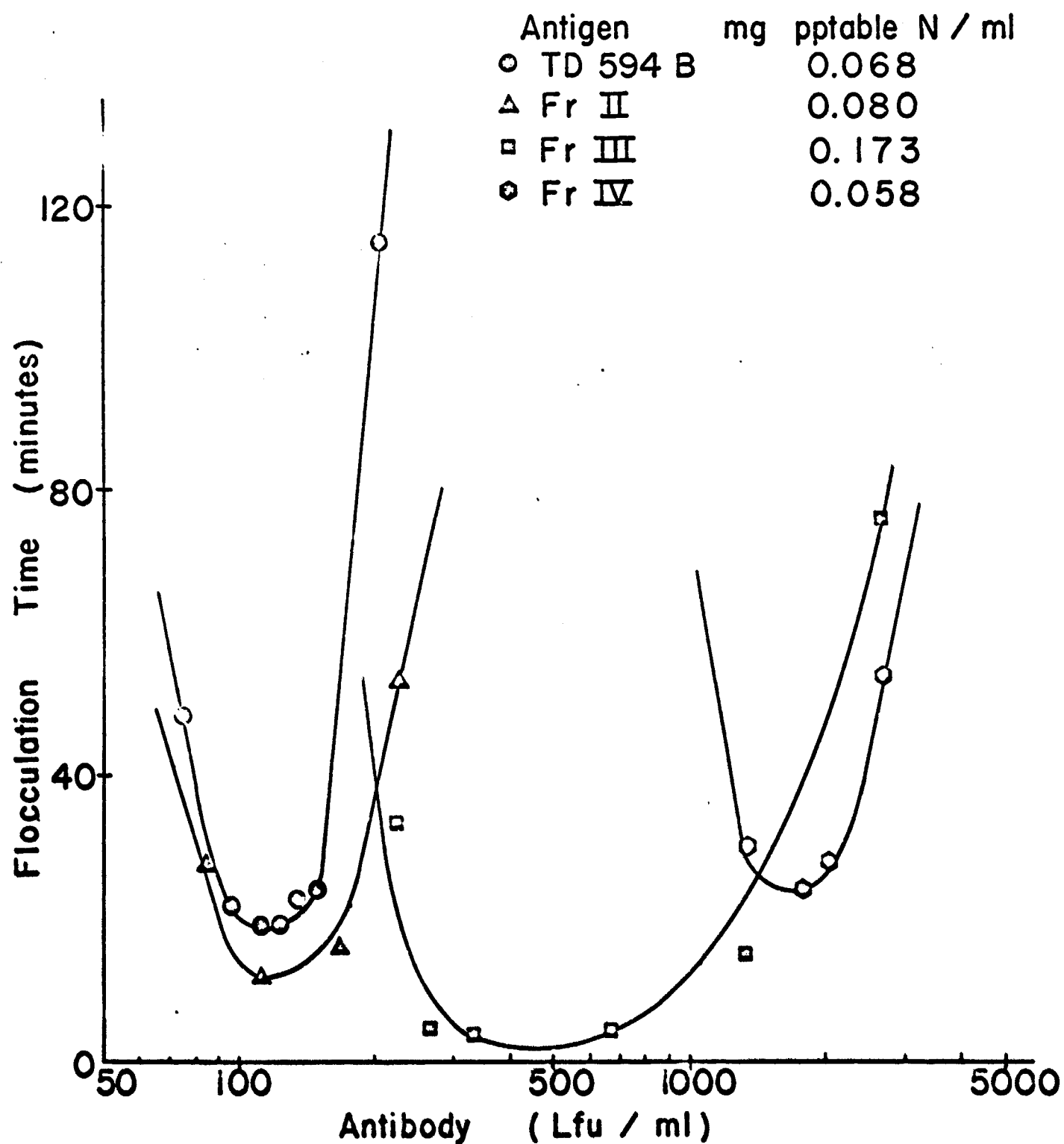


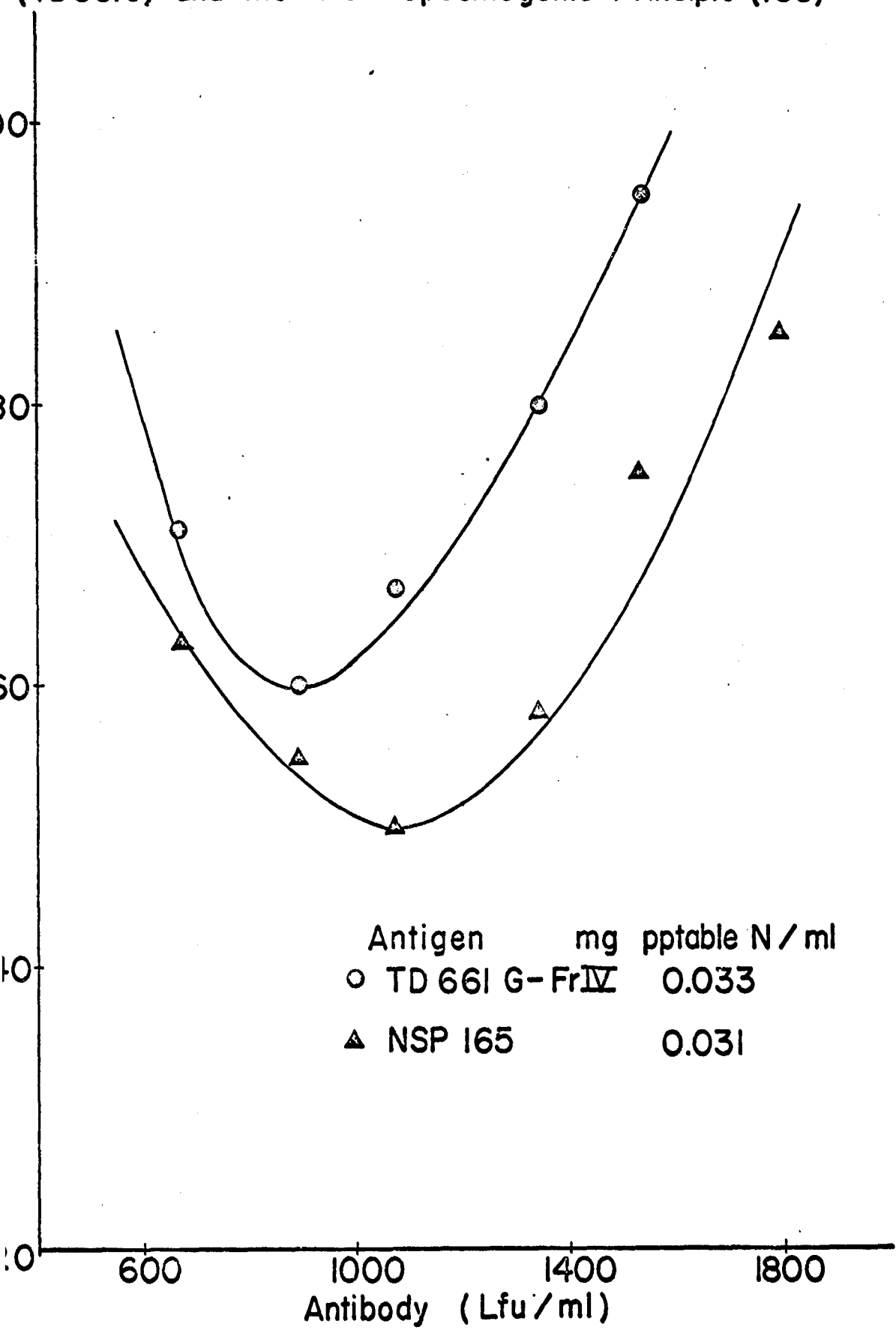
FIGURE 10 - FIGURE 12

Seroflocculation Curves

Seroflocculation of Crude Tetanus Toxin and Ammonium Sulfate Fractions II, III, and IV



Seroflocculation of Ammonium Sulfate Fraction IV (TD66IG) and the Non Spasmogenic Principle (165)



Seroflocculation of the Nonspasmogenic Principle and Ammonium Sulfate Fraction IV

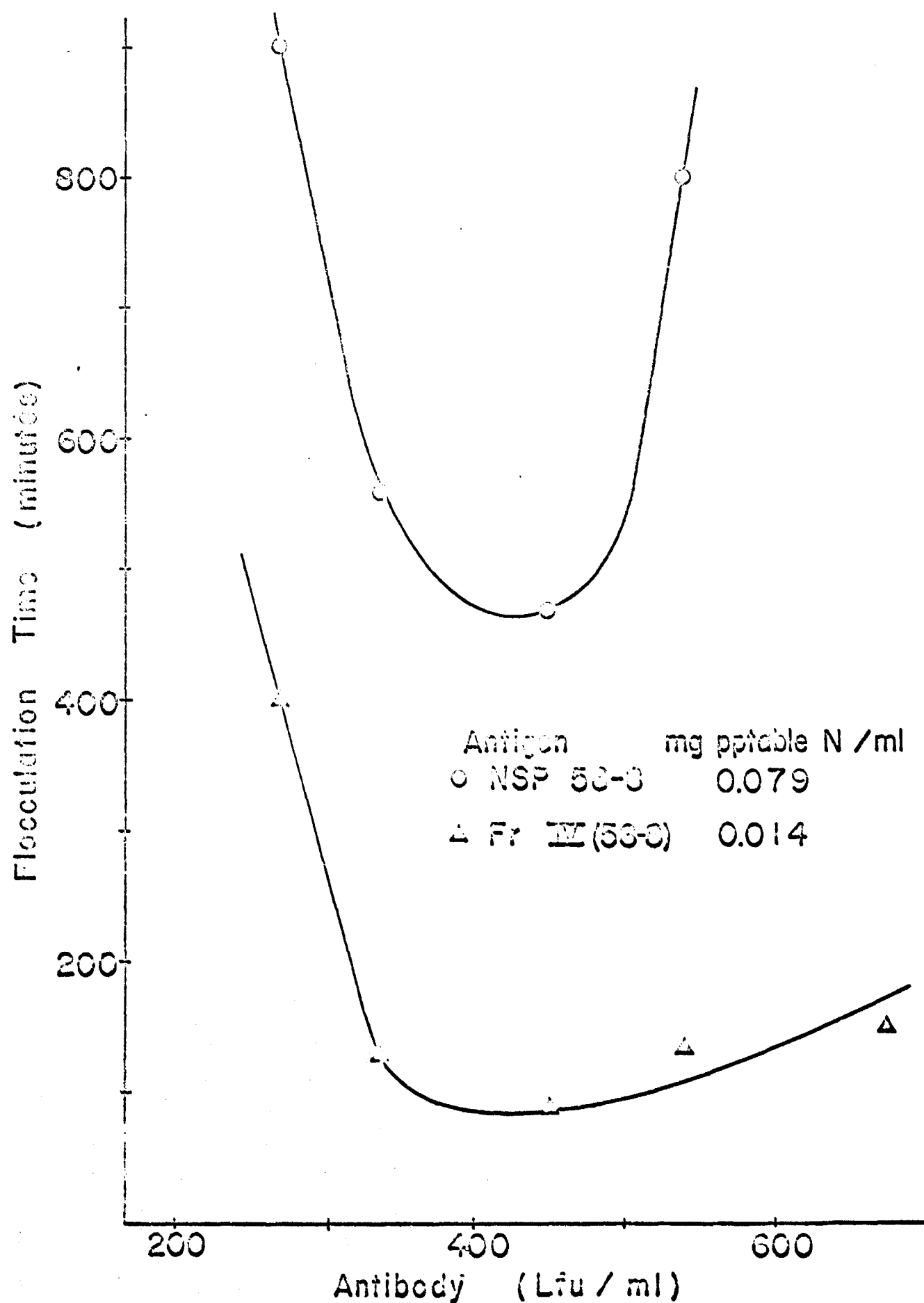


FIGURE 13

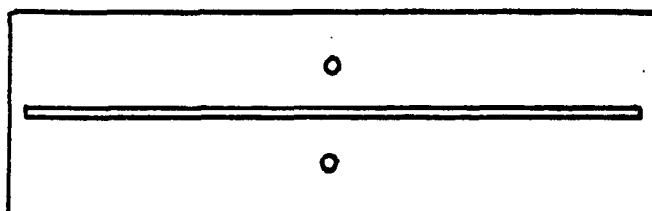
IMMUNOELECTROPHORESIS OF TD594B, TD594B FR. IV, NSP 58-8, AND NSP 58-8 FR. IV.

TOXIN PREPARATIONS AT 2.1 mg pptble N/ml

TETANUS ANTITOXIN AT 5.0 mg pptble N/ml

LEGEND

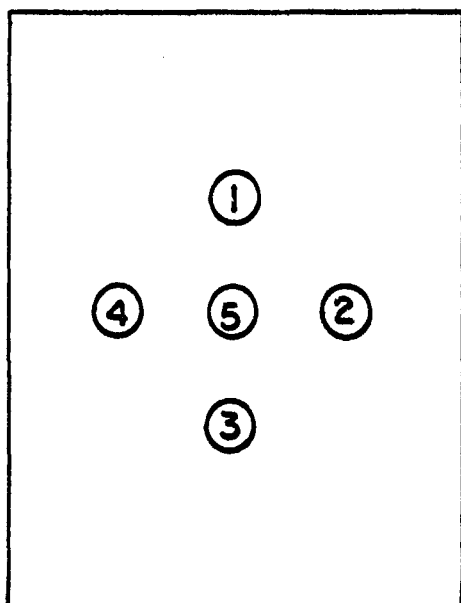
Immunoelectrophoresis



Antigens in wells

Antitoxin in center trough

Immunodiffusion



Antigens in depots 1-4

Antitoxin in depot 5



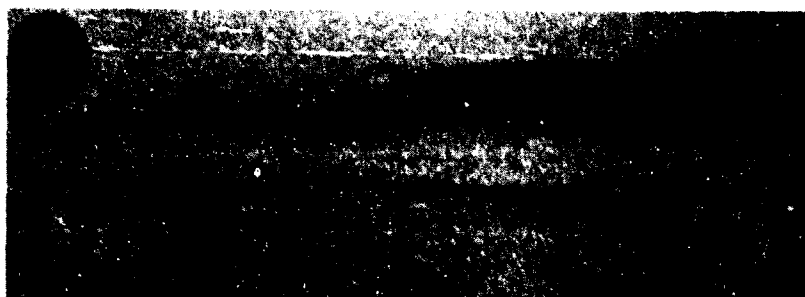
TD594B FR IV

TD594B



NSP 58-8

TD594B



NSP 58-8

NSP 58-8 FR IV

FIGURE 14

IMMUNOELECTROPHORESIS OF TD594B FR. IV, TD661G FR. IV, NSP 58-8 FR. IV,
NSP 58-8 FR. IV, NSP 165, TD594B, AND TP III

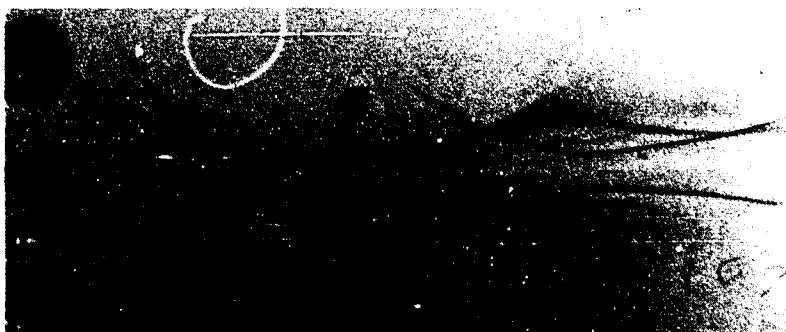
TOXIN PREPARATIONS AT 2.1 mg pptble N/ml except TP III
and TD594B at 1.6 mg pptble N/ml in slide 138

TETANUS ANTITOXIN AT 5.0 mg pptble N/ml



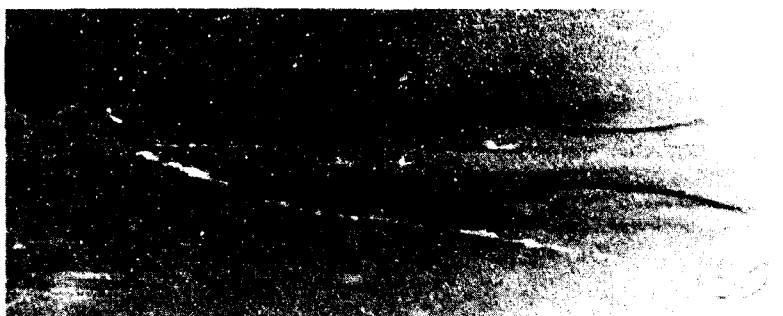
TD594B FRIV

TD66IG FRIV



NSP 165

TD66IG FRIV



NSP58 8 FRIV

NSP 165



TPIII

TD594B

FIGURE 15

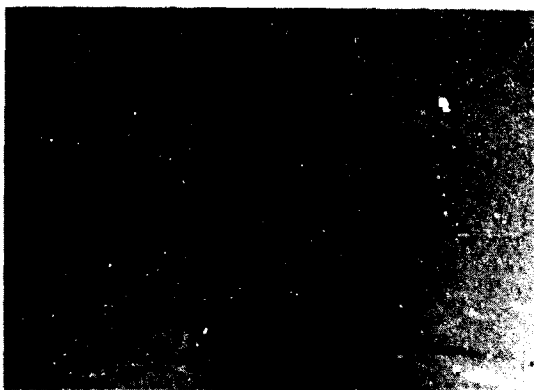
IMMUNODIFFUSION OF TD594B, NSP 58-8, NSP 58-8 FR. IV, AND TD661G FR. IV

TOXIN PREPARATIONS AT 2.1 mg pptble N/ml

TETANUS ANTITOXIN AT 5.0 mg pptble N/ml



1. TD594B
2. NSP 58-8
3. NSP 58-8 FRI
4. TD594B



1. TD 66IG FRI
2. TD 66IG FRI
3. TD594B
4. TD594B

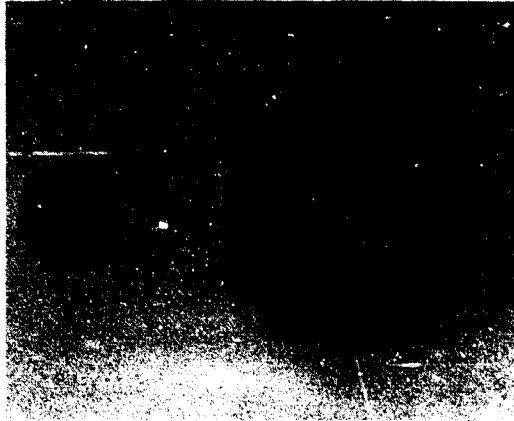
FIGURE 16

IMMUNODIFFUSION OF NSP165, TD661G FR. IV, TP III
AND TD594B

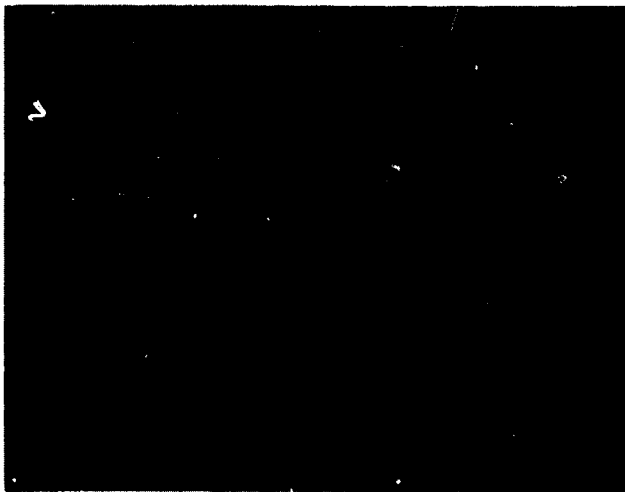
TOXIN PREPARATIONS AT 2.1 mg pptble N/ml in slide 88

TOXIN PREPARATIONS AT 1.6 mg pptble N/ml in slide 107

TETANUS ANTITOXIN AT 5.0 mg pptble N/ml



1. NSP 165
2. NSP 165
3. TD66IG FRI~~IV~~
4. TD66IG FRI~~IV~~



1. TP~~III~~
2. TD594B
3. TD594B
4. TD66IG FRI~~IV~~

FIGURE 17

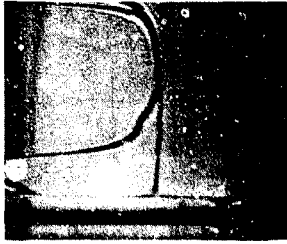
SEDIMENTATION VELOCITY PATTERNS OF TD594B AND TD594B FR. IV

TD594B AT 3.36 mg pptble N/ml

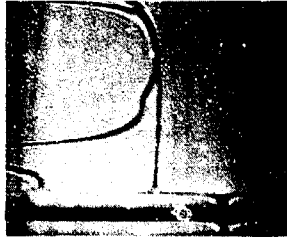
TD594B FR. IV at 0.33 mg pptble N/ml

TD594B and TD594B Fraction IV

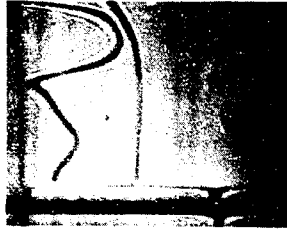
TD594B



32'



40'

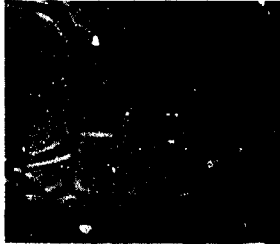


96'

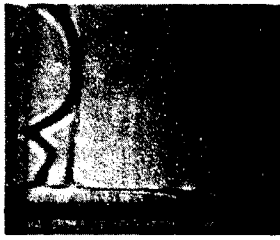


120'

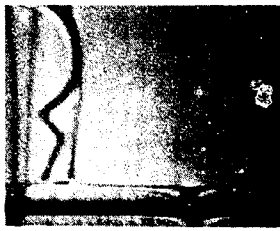
TD594 B
Fraction IV



32'



40'



80'



96'

FIGURE 18

SEDIMENTATION VELOCITY PATTERNS OF TD594B AND NSP 58-8

TD594B at 0.66 mg pptble N/ml

NSP 58-8 at 0.66 mg pptble N/ml

TD594B and NSP 58-8

TD594B



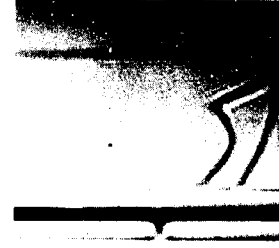
16'



24'

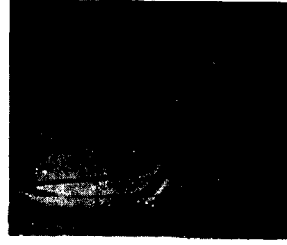


40'



80'

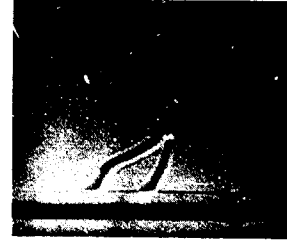
NSP 58-8



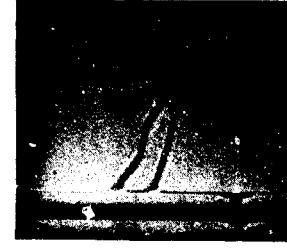
16'



24'



40'



80'

FIGURE 19

SEDIMENTATION VELOCITY PATTERNS OF NSP 58-8 AND NSP 58-8 FR. IV

NSP 58-8 at 0.66 mg pptble N/ml

NSP 58-8 Fr. IV at 0.33 mg pptble N/ml

NSP 58-8 and NSP 58-8 Fraction IV

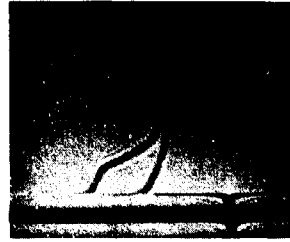
NSP 58-8



16'



24'



40'

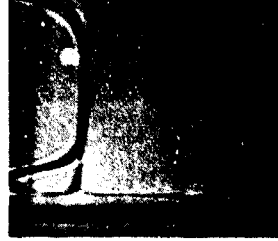


80'

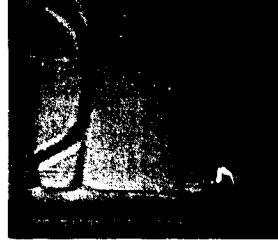
NSP 58-8
Fraction IV



16'



24'



40'



80'

FIGURE 20

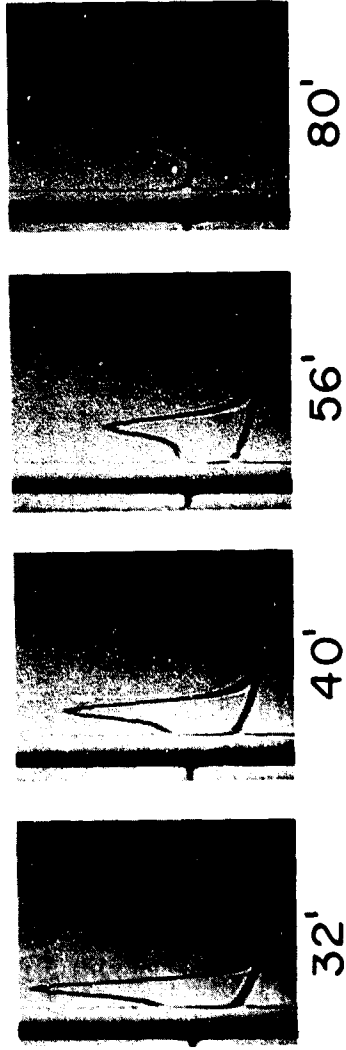
SEDIMENTATION VELOCITY PATTERNS OF TD661G FR. IV AND NSP 165

TD661G Fr. IV at 1.31 mg pptble N/ml

NSP 165 at 1.31 mg pptble N.ml

TD661G Fraction IV and NSP 165

TD661G Fr IV



NSP-165

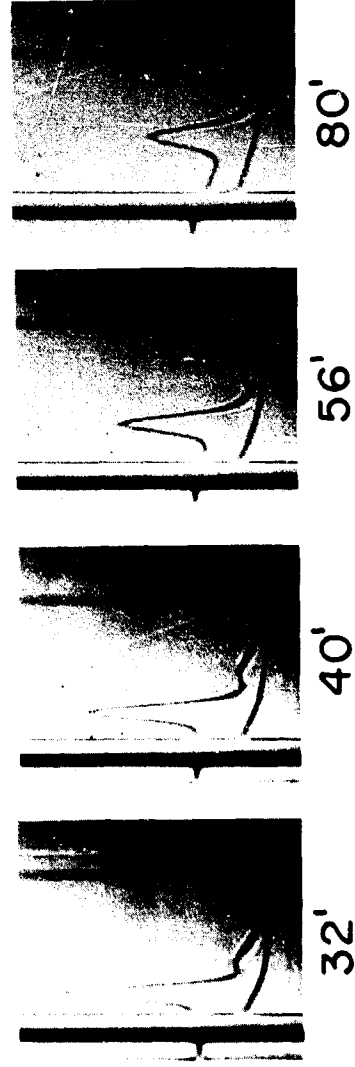


FIGURE 21

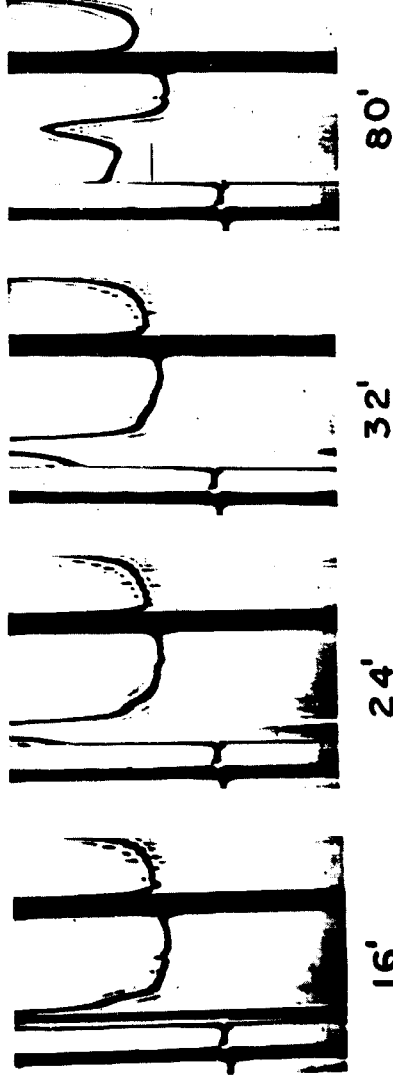
FIXED-PARTITION CELL SEPARATION OF NSP 165 AND SEDIMENTATION VELOCITY

RE-ANALYSIS

NSP 165 at 1.31 mg pptble N/ml

NSP 165 Separation Experiment

NSP 165
Fixed partition
cell separation



NSP 165
Sedimentation
Velocity
re-analysis

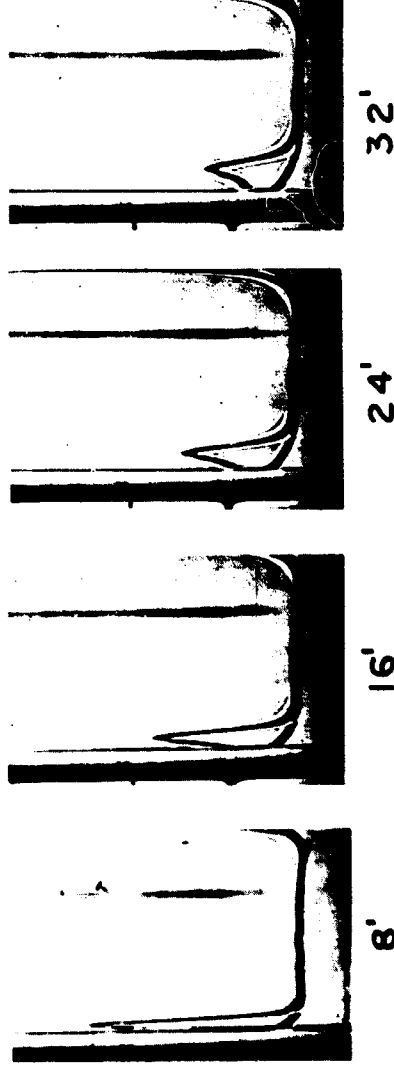


FIGURE 22

IMMUNOELECTROPHORESIS OF SEPARATED NSP 165 AND IMMUNODIFFUSION OF TD594B
AND TOXOID

NSP 165 Preparations at 1.31 mg pptble N/ml

TD594B at 1.6 mg pptble N/ml

TOXOID at 1.3 mg pptble N/ml

TETANUS ANTITOXIN at 5.0 mg pptble N/ml



Separated
NSP 165

Parent
NSP 165



1. TD594B
2. TD594B
3. TOXOID
4. TOXOID

FIGURE 23

SEDIMENTATION VELOCITY PATTERNS OF TP III AND TOXOID

TP III at 0.33 mg pptble N/ml

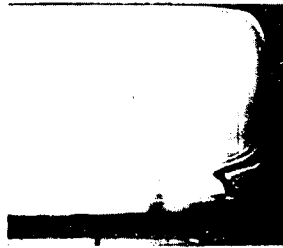
TOXOID at 0.66 mg pptble N/ml

TP III and Toxoid

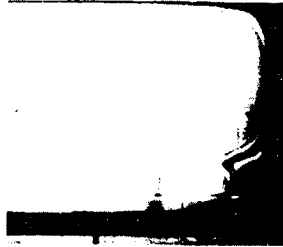
TP III



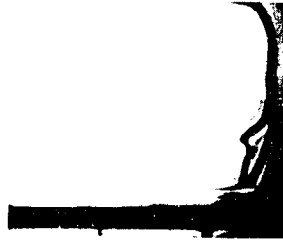
16'



24'

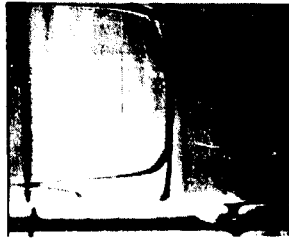


32'

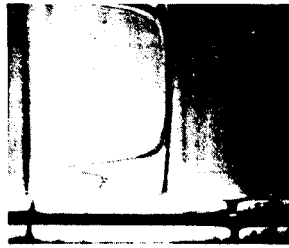


56'

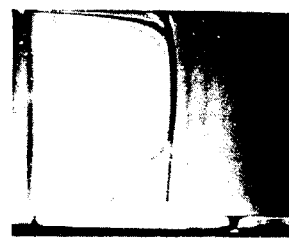
Toxoid



16'



24'



32'



48'

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1. ORIGINATING ACTIVITY (Corporate author) Stanford University, Stanford, California.		2a. REPORT SECURITY CLASSIFICATION None
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3. REPORT TITLE A Study of Immediate Sensitization by Adsorption of Antigens and Antibodies <u>In Vitro</u>		
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) Progress		
5. AUTHOR(S) (Last name, first name, initial) Feigen, George A.		
6. REPORT DATE May 16, 1966	7a. TOTAL NO. OF PAGES 91	7b. NO. OF REFS 18
8a. CONTRACT OR GRANT NO. NONR 225 (46) b. PROJECT NO. c. TASK No. 101-479 d.	9a. ORIGINATOR'S REPORT NUMBER(S) NONR 225 (46)	
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11. SUPPLEMENTARY NOTES None	12. SPONSORING MILITARY ACTIVITY Department of the Navy Office of Naval Research -- Biochemistry Branch Washington, 25, D.C.	
13. ABSTRACT <u>Sensitization</u> : Studies on the kinetics of sensitization have shown that the rate attains a maximum and depends on the antibody concentration and temperature. The lag period varies inversely as the temperature and inversely as the logarithm of antibody concentration. <u>Sea Urchin Toxin</u> : The active fraction is a proteolytic enzyme which attacks α_2 -globulins to produce a dialyzable kinin <u>Streptolysin</u> . Experiments on atrial action potentials show that the repolarization rate of single cells is slowed by streptolysin, suggesting that one of the effects of the toxin is on the Ach-ChE system of the heart. <u>Tetanus</u> : We have shown that the two physiological activities in crude tetanus toxin are properties of different molecules present in the mixture. A partial separation has been effected and the physical properties of the two factors have been determined: (1) Purification of the MEPP factor by the combined treatment of the crude preparation with ammonium sulfate and cerebroside-ganglioside enriches the electrophysiological potency 200-fold and decreases the lethal toxicity half a million-fold. (2) Partial purification of the tetanospasmin by methanol precipitation decreases the MEPP factor 90,000-fold and increases the lethal toxicity 6-fold. (3) The two factors can be separated immuno-electrophoretically. (4) The activity of the MEPP factor is concentrated with the 4S group of proteins and that of the lethal factor with the 7S proteins. (5) Estimates of the molecular weight show that the lethal factor has a value of 176,000 and that the MEPP factor has a value of about 59,000.		

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	ROLE	WT	ROLE	WT	ROLE	WT
Chemistry of Tetanus Toxin Effects of Tetanus Toxin, Streptolysin "O" Toxin Sea Urchin Toxin Mode of Action, Purification. Production of Dialyzable Kinin Streptolysin Toxin Effects on Cardiac Action Potentials Electrophysiology of						

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